SERUM AND FECAL MARKERS OF CHRONIC LYMPHOCYTIC ENTERITIS IN THE COMMON MARMOSET (*CALLITHRIX JACCHUS*)

A Dissertation

by

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Submitted to the Office of Graduate and Professional Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

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August 2018

Major Subject: Biomedical Sciences

ABSTRACT

Chronic lymphocytic enteritis (CLE) is a fatal condition in the common marmoset (*C. jacchus*) and is characterized by hypoalbuminemia. Fecal alpha$_1$-proteinase inhibitor (α_1-PI) concentrations can be used to diagnose enteric protein loss in humans, dogs, and cats. Marmoset serum α_1-PI was purified using immunoaffinity and ceramic hydroxyapatite chromatography, and partial characterization was performed. A radioimmunoassay (RIA) and a sandwich enzyme-linked immunosorbent assay (ELISA) were developed and analytically validated for the measurement of α_1-PI concentrations in serum and feces. Reference intervals for α_1-PI in serum and fecal extracts were established for both assays. Fecal α_1-PI concentrations were measured in marmosets with CLE. Serum cobalamin and folate concentrations serve as markers of gastrointestinal disease. Commercially available chemiluminescence immunoassays for the measurement of cobalamin and folate in human serum samples were analytically validated for the common marmoset. Serum concentrations of cobalamin and folate were measured in healthy marmosets and those with CLE. Mast cells have an important role in gastrointestinal disease. Fecal N-methylhistamine (NMH) concentrations, a breakdown product of histamine metabolism, was measured using a gas chromatography–mass spectrometry assay. Fecal NMH concentrations were measured in healthy marmosets and those with CLE.

The identity of purified marmoset α_1-PI was confirmed by peptide mass fingerprinting, N-terminal amino acid sequencing (EDPQGDAAQKMDTSHH), and by trypsin, chymotrypsin, and elastase inhibitory activity. Both the RIA and the ELISA developed were sensitive, linear, accurate, precise, and reproducible for the quantification of α_1-PI in serum and fecal extracts. However, the ELISA had limited linearity and accuracy for spiking recovery of fecal samples. The

RIA was used to measure fecal α_1-PI concentrations in 9 marmosets with a necropsy diagnosis of CLE. Fecal α_1-PI concentrations were not elevated in common marmosets with CLE and were not significantly different from healthy marmosets or those with other diseases. Low serum folate and cobalamin concentrations were moderately sensitive (>70%) and moderately specific (>70%) for CLE in marmosets. Fecal NMH concentrations were elevated in 7 of 8 marmosets with CLE and one marmoset with lymphoma and ulcerative enteritis. Increased fecal NMH concentrations and decreased serum cobalamin and folate concentrations may serve as a marker for CLE.

DEDICATION

In dedication to my wife and son for supporting me all the way, and to my mother, father, and sister who are a constant source of inspiration for pursuing my dreams.

ACKNOWLEDGEMENTS

I would like to thank my committee chair, Dr. Steiner, and my committee members, Dr. Suchodolski, Dr. Gaschen, Dr. Austad, and Dr. Tizard, for their guidance, patience, and support throughout the course of this research. A special thanks go to my committee chair who has been instrumental in getting me to Texas, helping me pursue my dreams in completing my Ph.D. and also giving me an opportunity to pursue additional clinical specialty training during the course of this program.

A special thanks goes to my collaborators Dr. Ross, Dr. Miller, and Dr. Tardiff without whose help this project would not have been possible, and also to Dr. Lidbury for all his help, support, and patience.

The project would not have been possible without the team at the gastrointestinal laboratory, particularly, Mrs. Cangelose and Mrs. Reed, the laboratory managers, the numerous service and research technicians, particularly Ms. Miller, graduate students, and student workers, many of whom have helped by running assays or by lending their support.

A special thank you also to my friends, colleagues, department faculty, and staff for making my Aggieland experience a great one.

Last, but certainly not least, a big thank you goes to my family and all my friends who have supported and encouraged me every step of the way, specially my mum for everything, and to my wife, Anitha for being a pillar of patience, support, and love.

CONTRIBUTORS AND FUNDING SOURCES

This work was supervised by a dissertation committee consisting of Dr. Joerg M. Steiner and Dr. Jan S. Suchodolski of the Department of Small Animal Clinical Sciences, Dr. Ian R. Tizard of Department of Veterinary Pathobiology, Dr. Frédéric Gaschen of the Department of Veterinary Clinical Sciences, Louisiana State University, and Dr. Steven N. Austad of the Department of Biology, The University of Alabama at Birmingham.

All work for the dissertation was completed independently by the student.

Graduate study was supported by a grant from the National Center for Research Resources, National Institute of Health # 1R24RR023344-01A2.

NOMENCLATURE

A_1AT	$Alpha_1$-antitrypsin
α_1-PI	$Alpha_1$-proteinase inhibitor
AGA	Anti-gliadin antibodies
AGP2A	Antibodies to glycoprotein 2
BMAC	Barshop marmoset aging center
BAPNA	N_α-Benzoyl-DL-arginine-p nitroanilide
BGS	Bone and gastrointestinal syndrome
CRP	C-reactive protein
CHT	Ceramic hydroxyapatite
^{51}Cr	Chromium-51
CLI	Chronic lymphocytic enteritis
CV	Coefficient of variation
CI	Confidence interval
CD	Cluster of differentiation
ELISA	Enzyme-linked immunosorbent assay
EPI	Exocrine pancreatic insufficiency
FPLC	Fast protein liquid chromatography
GC/MS	Gas chromatography–mass spectrometry
GI	Gastrointestinal
GALT	Gut-associated lymphoid tissue
HCl	Hydrochloric acid

IgA	Immunoglobulin A
IgG	Immunoglobulin G
IBD	Inflammatory bowel disease
IFCC	International federation of clinical chemistry
pI	Isoelectric point
kDa	Kilo dalton
LLOD	Lower limit of detection
$M\alpha_1$-PI	Marmoset alpha$_1$-proteinase inhibitor
MALDI-TOF-MS	Matrix-assisted laser desorption/ionization time of flight mass Spectrometry
MMP	Metalloproteinases
mAU	Milli-absorbance units
MWCO	Molecular weight cut-off
NMH	N-methylhistamine
NEPRC	New England primate research center
O/E	Observed over expected
PMF	Peptide mass fingerprint (PMF)
PLE	Protein losing enteropathy
RIA	Radioimmunoassay
RIAB	Radioimmunoassay buffer
Mr	Relative molecular mass
SDS–PAGE	Sodium dodecyl sulphate electrophoresis polyacrylamide gel electrophoresis
SNPRC	Southwest national primate research center

SD	Standard deviation
SAPNA	Succinyl-Ala-Ala-Pro-Phe-p-nitroanilide
SELDI-TOF-MS	Surface-enhanced laser desorption/ionization time of flight mass Spectrometry
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling
tTG	Tissue transglutaminase
Tris	Tromethamine
WMS	Wasting marmoset syndrome

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CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

1.1 The common marmoset in biomedical research

The common marmoset (*Callithrix jacchus*) is a Brazilian New World monkey that has been employed in biomedical research since the early 1960 and 1970's. However, its popularity in biomedical research has increased considerably over the years in North America and Europe.[1] Marmosets are gaining popularity because of their small size (350-450 g), reduced cost for maintenance, easy husbandry, easy habituation for routine clinical procedures, and decreased susceptibility to certain human zoonotic pathogens. Their shorter lifespan, ranging from 7 to 15 years, and early sexual maturity at an average age of about 1.5 years, also make them an ideal species for biomedical research. They also have a high reproductive efficiency with twin and triplet births making the self-perpetuation of a research colony easier.[1,90]

Currently, the marmoset is used for research in neuroscience, reproductive biology and teratology, infectious diseases, immunology, behavior, toxicology, and drug development and safety assessment.[90] The recent development of transgenic marmosets with germ line transmission[136] is further expected to increase their popularity in biomedical research. Marmosets are used as animal models for aging, stroke, multiple sclerosis, Parkinson's disease, Huntington's disease, Alzheimer's disease, and numerous human viral infections.[114] There is also a greater regulatory acceptance of toxicological data generated in the common marmoset compared to that generated with other species.[143] Marmosets are born as naturally occurring hematopoietic chimeras due to chorionic fusion during early gestational development, thus an individual marmoset has

tissues derived from self and sibling embryonic cell lines,[110] making them good models to study T and B cell-mediated diseases.

The popularity of marmosets in research has also increased due to shortage in availability of Rhesus macaques for research.[56] The common marmoset is also the first New World Non-Human primate after the chimpanzee, macaque, and orangutan whose complete genome has been sequenced.[33]

1.2 Chronic lymphocytic enteritis in the common marmoset

1.2.1 Wasting marmoset syndrome /disease, or chronic lymphocytic enteritis

Inflammatory diseases of the intestinal tract, particularly colitis, has been described in marmosets.[32,38,158] The first described set of marmosets with the 'wasting marmoset syndrome' (WMS) were reported at the Jersey Zoo in 1976.[75] Marmoset wasting disease / syndrome was described as a potentially fatal condition characterized by general debilitation, weakening, and loss of mobility with no identifiable etiology.[75] The term, WMS was initially used for a condition that would be observed in recently weaned marmosets,[139] but his since then being expanded to include the condition in marmosets of all ages.[124] WMS is the popular terminology among zoo and laboratory veterinarians as it aids in the description of the affected marmosets, who appear to waste away and become clinically debilitated and weak. At the same time, the name wasting disease / syndrome maybe misleading as the term "wasting disease" has also been used to describe conditions in other species in the animal kingdom that have an infectious origin attributed to prions in North American deer [165] or viruses in pigs.[4]

Recently, use of the term, inflammatory bowel disease (IBD), or more specifically chronic lymphocytic enteritis (CLE) has been advocated.[88] The nomenclature IBD/CLE is preferred in

marmosets as enteritis is the predominant lesion observed in numerous studies.[124,175] Lesions in marmosets with WMS are not restricted exclusively to the intestinal tract but also include lesions of other organ systems, such as the kidney, liver, pancreas, and the musculoskeletal and hematopoietic systems.

WMS has been reported in a number of marmoset species, but has been predominantly observed in the *Callithrix jacchus*.[63] Other species of marmosets such as the white-headed marmoset/ Geoffroy's marmoset (*Callithrix geoffroyi*), cotton-top tamarin (*Saguinus oedipus)* also demonstrate a similar spectrum of clinical signs but different diseases.[31,125]

A recent study used the term, bone and gastrointestinal syndrome (BGS) instead of WMS or CLE, because in most marmosets both systems are affected simultaneously.[11]

Inflammatory bowel disease in the human medical literature, refers to a chronic inflammatory condition of the distal small intestine and colon, specifically Crohn's disease, and ulcerative colitis as a result of an inappropriate inflammatory response to intestinal microbes in a genetically susceptible individual.[2] Extra-intestinal manifestations of IBD are common and primarily include the musculoskeletal and dermatological systems.[85] In the small animal veterinary literature, inflammatory bowel disease collectively refers to chronic enteropathy accompanied with both recurrent clinical signs and inflammation of the gastrointestinal tract. This may be the result of altered genetics, dysbiosis of the gut microbiota, immune system, environment, and/or diet.[142] Thus, the use of the term chronic lymphocytic enteritis (CLE) to describe the disease process in the common marmoset would be most appropriate.

1.2.2 Epidemiology of CLE in the common marmoset

CLE is seen in marmosets from zoological gardens and research colonies [63,154] in the United Kingdom,[32] Germany,[124] Brazil,[144] France,[18] United States of America,[154] and Japan.[108]

IBD, particularly CLE, has been a consistent finding in several *C. jacchus* colonies at two primate centers, the New England Primate Research Center (NEPRC), Southborough, MA and Southwest National Primate Research Center, Texas Biomedical Research Institute, San Antonio (SNPRC). Between 1991 and 2000, about 60.5% of all non-experimental marmosets at the NEPRC had some degree of IBD.[88] In the same center, IBD was observed in about 16% in marmosets less than 5.8 years and in 13% in those aged >5.8years between January 2004 to June 2009.[154] In a similar study from 2002-2011 at the SNPRC facility, 44% and 31% of deaths were attributed to IBD by looking at the causes of mortality for marmosets under the age of 6 and over the age of 6 years , respectively.[131] A disease prevalence between 4-6% among all age groups was seen at the German Primate Center from 1977-2000. At this center, marmosets with CLE were found to be between 5-7 years old. [124]. Fatality in recently weaned marmosets due to CLE has been reported to be about 4-5%.[139]

In one study, female marmosets were more frequently affected than makes.[124] However, no sex-related susceptibility has been reported in other studies.[88,139] Low birth weight, ancestry, or twinning could also not be associated with CLE in marmosets.[139]

1.2.3 Etiology and pathogenesis

To date, no definitive etiology for marmoset CLE has been identified. A variety of possible etiologies have been suggested, including intestinal pathogens, nutritional inadequacy, gluten food sensitivity, immune-mediated reaction, stress, pancreatic worms (*Trichospirura leptostoma*),

changes in the gut microbiota, digestive efficiency, level of chimerism, or a combination of several or even all of these factors.[68,88,130,152]

Marmosets in the wild feed on tree exudates, fruits, leaves, insects, and small vertebrates.[52] Nutritional inadequacy, particularly dietary protein deficiency has been described in the common marmoset. Clinical signs were alleviated by supplementation of milk protein in those marmosets.[19] In recently weaned marmosets, high protein foods in the diet also reduced clinical signs of CLE.[139] Circulating antibodies (IgG class) against cereals, citrus fruit, milk, and egg proteins have been reported to be present in marmosets,[20] suggesting the possible etiology of dietary sensitivity. Also, elevated IgA-antigliadin antibodies were also seen in this study.

Celiac disease in humans is associated with a wide range of presentations and includes an increased immunological responsiveness to dietary prolamins, a class of proteins found in wheat, barley, rye etc. Gluten sensitivity has been suspected in marmosets as a cause of CLE.[52] Increased levels of IgA-gliadin antibodies (IgA-AGA) and of IgA-containing circulating immune complexes (IgA-CIC), have been reported in marmosets[137] with signs of wasting disease compared to healthy marmosets. A French study failed to identify changes in serological markers in marmosets with CLE.[18] Only one out of the 9 cases of marmoset with CLE had histopathological lesions comparable to human celiac disease.[18] In a more recent study, serum IgA antibodies directed against gliadin (AGA), tissue transglutaminase (tTG), and glycoprotein 2 (AGP2A) were measured in marmosets on gluten free diets. The addition of gluten to the diet increased AGA, tTG, and AGP2A concentrations in some marmosets, and decreased AGA and AGP2A after removal of gluten. When marmosets where on the gluten containing diet, more episodes of diarrhea were reported. Improved body weights were observed when marmosets were fed gluten-free diets,[84] however the response was not uniform in all the marmosets in the study.

CLE in the common marmoset was presumed to be similar to human celiac disease. When marmosets were fed a gluten free (recuperation) diet, they showed weight gain. However, when the normal colony diet was resumed, clinical signs did not recur. This is in contrast to humans with celiac disease in whom a gluten free diet is a lifetime requirement and even tiny amounts of gluten may cause severe clinical signs.[88] In one study, nutritional intervention induced reversal of CLE in the marmoset.[36] Histopathology of human patients with celiac disease is also highly variable and can show lesions that are comparable to this in marmosets with CLE.[23,113] Definitive diagnosis of celiac disease in humans requires clinical signs, serology (presence of antitissue transglutaminase antibodies), and/or biopsy. Demonstration of improvement, between initial diagnosis when the person is on a gluten containing diet is necessary, and after therapy is necessary for confirmation.[23,134] A more recent study showed that in zoological gardens, clinical signs persisted despite the use of gluten free diets. However, in one study the switch to a hypoallergenic diet did result in resolution of clinical signs.[27]

Trichospirura leptostoma is a pancreatic nematode in the common marmoset. Marmosets become infected through ingestion of common cockroaches, which serve as the intermediate host. Clinical signs of infection mimic signs in marmosets with CLE.[12,121] However, with modern husbandry practices in research colonies exposure to cockroaches is very unlikely.

Changes in the fecal microbiota have been reported in marmosets with signs of CLE, particularly with respect to changes in anaerobic bacteria.[86] However, another study was unable to discriminate the microbiota between marmosets with intestinal inflammation and those without intestinal inflammation.[7] A more recent study using 16S rRNA sequencing techniques, identified changes in composition of bacterial groups between a specific pathogen free (SPF) barrier primate colony and a conventional colony, where no evidence of gastrointestinal disease was reported in

the SPF colony.[130] Altered bacterial groups included *Bifidobacterium* and *Fusobacterium B*, with the conventional colony having a comparatively high abundance of *Fusobacterium B* and a low abundance of *Bifidobacterium*, in contrast, to the SPF colony which had a higher abundance of *Bifidobacterium* and lower abundance of *Fusobacterium B.*

Marmosets with CLE showed higher levels of chimerism based on quantitative Y-chromosome PCR in affected tissues such as the jejunum. This increased level of chimerism was attributed to infiltrating chimeric lymphocytes contributing to tissue chimerism.[152] However, a loss of immune tolerance or an immune mediated mechanism is still possible.

A recent study suggested that gastrointestinal inflammation, leading to impaired digestive efficiency and subsequent malabsorption of critical nutrients, leads to clinical signs of CLE.[68,175]

A specific etiology has not been identified, and currently CLE is considered to be multifactorial in origin with exogenous and endogenous factors leading to an altered intestinal immune system, similar to the mechanisms suspected in humans and dogs.

1.2.4 Clinical signs

The disease is characterized by diffuse to segmental lymphocytic enteritis. Clinical signs reported include weight loss, progressing to emaciation, with loss of about 25–50% of body weight.[31] A body weight <325 g or a weight loss >0.05% of peak weight per day has been associated with CLE.[11] Muscle atrophy, progressive muscular weakness, stiff hind limb gait, progressing to hind limb paralysis[12] have also been reported. Diarrhea may or may not be present and when present diarrhea may be transient, or chronic (greater than 2 weeks), with high-viscosity.[108] The hair coat can be in poor condition, and tail alopecia can also be seen.[84,87] Juveniles

fail to thrive, and there is a generalized weakness in weanlings.[139] Anorexia and selective eating have also been reported.[75,86]

1.2.5 Clinical pathology

The most common finding in marmosets with CLE is macrocytic, normochromic anemia and hypoalbuminemia.[87] Other findings include elevated liver enzyme activities (i.e., ALP, ALT, AST)[12] and muscle enzyme activity (CK),[139] elevated platelet counts,[87] and possible leucocytosis.[108] Low albumin concentrations have been reported in multiple studies.[115,175] An albumin concentration <3.5 g/dL followed by a body weight <325 g was able to identify 100% of the marmosets affected with concurrent bone and gastrointestinal disease.[11] However, in another study, serum albumin concentrations failed to differentiate between healthy marmosets and those with CLE.[108]

1.2.6 Diagnosis

Currently, an ante-mortem diagnosis of CLE in the marmoset is made based on clinical signs, a history of weight loss, and a decreased serum albumin concentration.[11] Clinical criteria also include non-responsiveness to common antibiotic therapy or evidence of relapse after short-term therapeutic success, and a normal appetite.[175] The gold standard for the diagnosis of CLE is histopathological confirmation of lymphoplasmacytic enterocolitis.[88]

A histopathological classification of enteritis based on severity (i.e., negative, minimal, mild, moderate, or severe) has been described. This classification takes into account the composition of the inflammatory cells (i.e., mononuclear or polynuclear) in the lamina propria, crypt abscesses, GALT hyperplasia, and the extent of ulceration.[175] Histopathology findings

include marked shortening of villi, hyperplasia of the crypt epithelium, lymphocytic infiltrates (i.e., T cells, CD3 CD8-positive lymphocytes) that can expand and replace the lamina propria, which can be associated with a complete loss of villus architecture ultimately leading to clinical signs.[88]

1.2.7 Differential diagnosis

Any disease that leads to progressive weight loss in the common marmoset should be differentiated from CLE. Such diseases include neoplastic diseases (i.e., spontaneous malignant T cell lymphoma,[169] small intestinal adenocarcinoma,[100] infectious diseases (i.e., Epstein–Barr-related herpesvirus,[126] *Mycobacterium tuberculosis*,[28] helicobacter infections[138]), parasitic diseases (i.e., *Giardia sps*.,[81] *Platynosomum sps*[144]), degenerative diseases (i.e., renal disease,[19,64] pancreatic diseases[19]), systemic inflammatory diseases (i.e., amyloidosis[89]), and environmental stressors.[81] CLE may also be seen concurrently with renal disease or systemic amyloidosis, making the diagnosis more challenging.

1.2.8 Markers for CLE

Newer markers such as fecal calprotectin,[108] serum matrix metalloproteinases,[171] serum IgA antibodies to gliadin, and related proteins [84] have recently been investigated.

Calprotectin is a major protein in neutrophilic granulocytes and fecal calprotectin assays have been used to predict intestinal inflammation in human patients with chronic enteritis. The concentration of fecal calprotectin is directly proportional to inflammatory neutrophil migration towards the intestinal tract and it is believed to induce secretions of various chemokines and the promotion of apoptosis.[46,173] Fecal concentrations of calprotectin have been explored in the

common marmoset. One study showed no alterations in fecal concentrations of calprotectin in marmosets with CLE.[11] However, another study showed marked increases in the fecal concentrations of calprotectin in marmosets with CLE.[108] Both studies included a small number of marmosets. However, the latter study examined the expression pattern of calprotectin using immunocytochemistry to distinguish between marmosets with CLE and healthy controls.[108] Calprotectin expression was seen in the extravascular space of the colon in marmosets with CLE. A terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay for the detection of DNA fragmentation generated during apoptosis was also increased in the marmosets with CLE in this study.

Metalloproteinases (MMPs) are a group of proteolytic enzymes that are involved in the remodeling and degradation of extracellular matrix. In humans, the overexpression of metalloproteinases and weaker expression of their inhibitors have been implicated in the development of IBD.[67] In humans, measurement of MMP in serum has been reported to help in distinguishing between active and inactive IBD.[92] Increased serum MMP9 concentrations have been reported in marmosets with clinical signs of CLE,[171] and these concentrations have also been shown to decrease after therapy with tranexamic acid, which coincides with an improvement in body weight, hematocrit, albumin, and other parameters.[172]

Changes in serum concentrations of IgA antibodies to gliadin and related proteins as described before have also been suggested as markers of CLE.

C-reactive protein (CRP) is an acute phase reactant that can be measured in serum and can be used to detect systemic inflammatory disease, including gastrointestinal (GI) disease, in humans and dogs.[5,105] CRP has also been shown to be an acute phase protein in the marmoset.[135] However,

CRP concentrations were not found to be significantly different between marmosets with and without CLE.[11]

Secretory IgA, a fecal indicator of infectious enterocolitis that has also been used as an indirect marker of mucosal immunity was also studied in marmosets with CLE and healthy controls and no significant changes were observed between these two groups in that particular study.[11]

Other non-invasive markers, such as serum anti-saccharomyces cerevisiae antibody (ASCA), anti-tissue transglutaminase antibody, perinuclear anti-neutrophil cytoplasmic antibody (pANCA), and fecal serotonin used for the diagnosis, classification, or monitoring of gastrointestinal disease in human medicine and in some veterinary species were studied in the marmoset using commercially available assay kits for humans. However, no cross-reactivity was appreciated when using these assays.[11]

1.2.9 Treatment

To date, no proven effective treatment for CLE in the marmoset has been described, as the final diagnosis is usually made at necropsy.[88] Earlier studies have shown that therapeutic measures often only lead to temporary improvement and relapses are common.[31,63]

Treatment with corticosteroids[115] or tranexamic acid[172] has been described. In marmosets with a body weight ≤325 g and a serum albumin ≤3.5 g/dl for at least 2 weeks, prednisone at 1 mg/kg, or adjusted budesonide therapy at a starting dose of 0.5 mg per animal for 8 weeks, improved body weight, and serum albumin concentrations. However, no confirmation of CLE lesions on histolopatholgy was performed in that study.[115] Similar results have not been reported from other centers.[172]

Tranexamic acid is a plasmin inhibitor that has hemostatic and anti-inflammatory effects. Plasmin is an enzyme in the fibrinolytic cascade, that activates matrix metalloproteinases, (MMP), particularly MMP9, which has been reported to be increased in marmosets with CLE. In one study, 6 marmosets with clinical signs of CLE received tranexamic acid therapy [0.5 ml of a 1% solution administered intraperitoneally once daily], amino acid supplementation (3 mL into the saphenous vein 3 times each week), iron supplementation (0.1 mL, administered orally daily), and rehydrating fluid therapy (5 ml of Ringer's lactate solution with 0.5 mL of a vitamin formulation injected subcutaneously 3 times each week) for 8 weeks, followed by a 4 week follow up period. This treatment protocol was associated with an increase in body weight, improved anemia (i.e., increased hematocrit), increased serum albumin concentration, decreased MMP9 concentration, and resolution of tail alopecia in the 4 week follow up period.[172]

However, a curative treatment for CLE in the common marmoset has not been described.

1.3 Protein losing enteropathy

1.3.1 Pathogenesis

Mechanisms for enteric protein loss include lymphatic obstruction, mucosal disease with erosions or ulcerations, or increased mucosal permeability as a result of cell damage or death.[155]

1.3.2 Alpha$_1$ proteinase inhibitor

Alpha$_1$ proteinase inhibitor (α_1-PI) is a serum glycoprotein synthesized by the liver [80] that is released into the systemic circulation and inhibits a variety of serine proteases, protecting tissues from enzymatic damage. Recently, α_1-PI has also been described in modulating immunity, inflammation, proteostasis, apoptosis, and possibly cellular senescence.[61] Alpha$_1$ proteinase

inhibitor has been purified in many species, including humans,[116] dogs, cats,[44] sheep,[102] goats,[161] rabbits,[79] mice,[101] rats,[83] guinea pigs,[151] Rhesus monkeys,[17] and opossums.[30] Across different species the structure and function of α_1-PI are similar. However, a relative deficiency of α_1-PI has only been reported in people with certain genetic genotypes.[60] Deficiency has also been suspected in a single dog.[98] Limited cross-reactivity for α_1-PI has been shown between species.[129]

Alpha$_1$ proteinase inhibitor is found in small concentrations within the lumen of the gastrointestinal tract. Alpha$_1$-PI has a molecular weight that is similar to that of albumin it is believed to be lost into the intestinal lumen at a rate comparable to that of albumin loss. However, unlike albumin, α_1-PI is resistant to enzymatic and bacterial degradation within the lumen of the gut, allowing for it to be detected in the feces by an immunoassay.[96] Fecal α_1-PI concentrations may be increased even before hypoalbuminemia is observed,[150] making it an early marker for protein losing enteropathy (PLE). The use of fecal α_1-PI as a marker for gastrointestinal protein loss has been described as early as 1977 in humans.[37] One consistent finding in marmosets with chronic lymphocytic enteritis is hypoalbuminemia, which is believed to be the result of enteric protein loss. Thus, α_1-PI concentrations in feces might help to serve as a surrogate marker of enteric protein loss in this species. Currently, analytically and clinically validated assays are available for use in humans,[72] dogs,[58,96] and cats.[26]

Increased fecal concentrations of α_1-PI have been reported in dogs with gastrointestinal disease, particularly IBD and lymphangiectasia,[107] acute hemorrhagic diarrhea syndrome,[57] parvoviral gastroenteritis,[104] tylosin responsive diarrhea,[164] and also in healthy sled dogs after a race.[39] In cats, increased fecal concentrations of α_1-PI have been reported in patients with severe IBD or confirmed gastrointestinal neoplasia.[25] In humans, increased fecal concentrations have been reported in a variety of gastrointestinal disorders,[149]

and also has been described as an indicator of Crohn's disease activity.[99] A good correlation has been reported between the gold standard, ^{51}Cr-albumin excretion and fecal α_1-PI in humans [123] and dogs.[94]

1.4 Other non-invasive markers of gastrointestinal health

1.4.1 Serum cobalamin and serum folate concentrations

Cobalamin (i.e., vitamin B12) is a water soluble vitamin that is an essential co-factor for methylmalonyl CoA mutase. Intestinal absorption of cobalamin is comprised of a multistep mechanism, with absorption occurring at receptors on ileal enterocytes[91,150] Cobalamin deficiency is reported frequently in dogs and cats with exocrine pancreatic insufficiency, distal small intestinal disease, diffuse small intestinal disease involving the distal small intestine, or genetic defects in certain dog breeds.[47] Exocrine pancreatic insufficiency (EPI) causes hypocobalaminemia in dogs as the majority of intrinsic factor in dogs is synthesized by pancreatic acinar cells,[9,35] which are non-functional in dogs with EPI. Ileal disease is believed to lead to damage or decreased expression of cobalamin receptors, ultimately leading to reduced cobalamin absorption and deficiency once cobalamin stores in the body have been used up. Intestinal dysbiosis can also lead to decreased serum cobalamin concentrations.[49] The reported prevalence of cobalamin deficiency in dogs and cats with gastrointestinal disease ranges from 6-18.5% in dogs[5,35] and from 17-61% in cats,[128,141] respectively. Furthermore, hypocobalaminemia has been associated with a negative outcome in dogs with chronic enteropathy[5] or exocrine pancreatic insufficiency[8], and cobalamin supplementation may have a therapeutic potential.[132] To the best of our knowledge, no data on cobalamin status in marmosets with CLE have previously been reported.

Folate (i.e., vitamin B9) is also a water-soluble B complex vitamin. Dietary folate in the form of folate polyglutamate is poorly absorbed in the intestinal tract. However, the enzyme, folate deconjugase in the jejunal brush border converts folate polyglutamate into monoglutamines, which then get absorbed into the enterocytes of the proximal small intestine utilizing specific carriers. Chronic intestinal disease damages folate carriers, reducing their uptake, utilization of body stores of folate, and ultimately leading to decreased serum concentrations of folate.[146] Increased serum concentrations have been reported in dogs with intestinal dysbiosis.[49] In addition, chronic enteropathy also can lead to decreased serum folate concentrations in dogs and cats.[35,128] Changes in serum cobalamin and/or folate concentrations can serve as surrogate markers for the presence of small intestinal disease,[49] especially because dietary deficiency of cobalamin and folate is highly improbable and it is believed that even withholding food for several weeks duration does not cause serum cobalamin and folate concentrations to become subnormal.[146]

1.4.2 N-Methylhistamine

Mast cells have been described in the gastrointestinal mucosa of dogs[50] and humans[127] and have been investigated in patients with chronic enteropathy in dogs[6,13] and inflammatory bowel disease[111] in humans. However, mast cells can be missed during evaluation of sections routinely stained with hematoxylin and eosin. Their detection requires either special staining with toluidine blue or immunohistochemistry for mast cell tryptase.[6,13] Markers for mast cell activation rather than their absolute numbers seen on histopathology, have also been suggested as a better method to measure their activity. Histamine is primarily stored in mast cells and serves as a marker of mast cell degranulation. Rapid metabolism of released histamine leads to the formation of N-methylhistamine (NMH) and imidazole acetaldehyde.[153] Fecal concentrations of NMH were found

to be increased in a subset of dogs with chronic enteropathy, suggesting that mast cell- mediated inflammation plays a role in this condition.[6,13] However, the role of mast cells has not been investigated in marmoset CLE.

1.5 Biomarkers

1.5.1 Characteristics for an ideal biomarker

Markers for the diagnosis and monitoring of marmoset CLE that have a clinical utility have not yet been established as evidenced from the studies mentioned before. Non-invasive early markers for disease, using blood, urine, or feces would be ideal as opposed to intestinal biopsies which in the marmoset can only be obtained surgically or at necropsy. Ideally, these tests should be fast, easy to perform, and also facilitate screening of a large number of animals.

1.5.2 Diagnostic tests: immunoassays

Immunoassays are based on signal responses generated from an antibody–antigen interaction. The response is generated from either an enzymatic, luminescent, or radioisotopic label attached to the antigen or antibody, or to a secondary binding reaction. The concentration of the unknown analyte is calculated from a standard curve.[45] Immunoassays such as radioimmunoassays (RIA), enzyme-linked immunosorbent assays (ELISA), and enzyme immunoassays (EIAs) are routinely used in diagnoses and have been described since the mid-1900s.[53]

Any immunoassay has to be analytically validated before routine use. Analytical validation involves determining the linearity, accuracy, precision, reproducibility, sensitivity, robustness, and stability of the analyte to ensure that immunoassay is suitable for its intended purpose.[160]

1.6 Hypotheses and research objectives

1.6.1 Hypotheses

The hypotheses of this study are:

1. Common marmosets with chronic lymphocytic enteritis have protein losing enteropathy as evidenced by an increased fecal α_1-PI concentration.
2. Common marmosets with chronic lymphocytic enteritis will have altered serum concentrations of cobalamin and folate.
3. Common marmosets with chronic lymphocytic enteritis will have evidence of mast cell degranulation in the gastrointestinal tract.

1.6.2 Research objectives

The objectives of this study are

(1) To purify and partially characterize α_1-proteinase inhibitor from the serum of the common marmoset.

(2) To develop and analytically validate a radioimmunoassay for the measurement of α_1-proteinase inhibitor concentrations in serum samples and fecal extracts from the common marmoset.

(3) To develop and analytically validate an enzyme linked immunosorbent assay for the measurement of α_1-proteinase inhibitor concentrations in serum samples and fecal extracts from the common marmoset.

(4) To evaluate α_1-proteinase inhibitor concentrations in fecal extracts from healthy marmosets and marmosets with chronic lymphocytic enteritis.

(5) To measure serum concentrations of cobalamin and folate in healthy marmosets and marmosets with chronic lymphocytic enteritis.

(6) To measure fecal concentrations of N-methylhistamine in healthy marmosets and marmosets with chronic lymphocytic enteritis.

CHAPTER II

PURIFICATION AND PARTIAL CHARACTERIZATION OF α_1-PROTEINASE INHIBITOR IN THE COMMON MARMOSET (*CALLITHRIX JACCHUS*)*

2.1 Introduction

The common marmoset (*Callithrix jacchus*) is a Brazilian new world monkey that has been employed in biomedical research since the early 1960's. Its popularity has increased considerably over the years in North America and Europe.[1] Marmosets are utilized because of their small size, reduced cost for maintenance, easy husbandry, rapid reproductive turnover, and decreased susceptibility to certain human pathogens. Currently they are used for research in neuroscience, reproductive biology, infectious diseases, behavioral science, drug development, and safety assessment, as well as models of aging research.[90] Recently, a transgenic marmoset with germ line transmission has been developed,[136] which holds great potential for further biomedical research. Inflammatory diseases of the intestinal tract, particularly colitis, have been described in marmosets.[32,38,158] Inflammatory bowel disease (IBD), particularly chronic lymphocytic enteritis (CLE), has been a consistent finding in *C. jacchus* colonies as evidenced by necropsy findings at two primate centers, the NEPRC and SNPRC. Between 1991 and 2000 approximately 60.5% of all marmosets used as controls in various studies had some degree of IBD at necropsy at the NEPRC. Also, between January 2004 and June 2009, IBD was observed in about 16% of marmosets who were >1year of age at the NEPRC.[154] In a similar study at the SNPRC facility from 2002 to 2011, IBD was attributed as the cause of death in 44% of marmosets below the age

*Reprinted with permission from Parambeth JC, Suchodolski JS, Steiner JM, 2015.Purification and partial characterization of α_1-proteinase inhibitor in the common marmoset (*Callithrix jacchus*). Research in Veterinary Science 2015 99:17-22. Copyright (2015) by Elsevier.

of 6 years.[131] While no specific etiology has been identified, many etiological factors such as gluten sensitivity, dietary protein deficiency, and the pancreatic spirurid nematode (*Trichospirura leptostoma*) have been reported.[88] The disease is characterized by diffuse to segmental lymphocytic enteritis, and clinically manifests itself by failure to thrive in juveniles, or weight loss in adults, with or without diarrhea. A diagnosis is made based on clinical signs, a history of weight loss, and clinicopathological findings like a decreased serum albumin concentration. No effective treatment exists and a final diagnosis is usually made only at necropsy.[88]

Alpha$_1$-proteinase inhibitor (α_1-PI) is a serum glycoprotein synthesized by the liver,[80] released into systemic circulation, and inhibiting a variety of serine proteases that protect various tissues from enzymatic damage. Recently, α_1-PI has also been described as being of importance in modulating immunity, inflammation, proteostasis, apoptosis, and possibly cellular senescence.[61] Alpha$_1$-proteinase inhibitor has been purified from many species including human,[116] dog,[97] cat,[44] sheep,[102] goat,[161] rabbit,[79] mouse,[101] rat,[83] guinea pig,[151] Rhesus macaque,[17] and opossum.[30] Across species, the protein structure and function remain similar, however, a relative deficiency has been reported in people with certain genetic genotypes.[21] Deficiency of α_1-PI has also been reported in a single dog.[98] However, there is limited cross-immuno-reactivity for α_1-PI between species and thus assays for the measurement of α_1-PI are species-specific.[129]

Under physiologic conditions only minimal amounts of alpha$_1$-proteinase inhibitor are found within the lumen of the gastrointestinal tract. Having a molecular weight similar to albumin, it is believed to be lost into the gastrointestinal tract at a rate comparable to that of albumin. However, unlike albumin, α_1-PI is resistant to bacterial degradation or the effects of digestive enzymes within the lumen of the gut, enabling it's detection in fecal samples by an

immunoassays.[96] Fecal α_1-PI may be increased even before hypoalbuminemia is observed, making it an early marker for intestinal protein loss.[159] Validated assays for measurement of fecal alpha$_1$-proteinase inhibitor for detection of intestinal protein loss are available for humans,[72] dogs,[58,96] and cats.[26]

Marmoset α_1-PI has not yet been purified or characterized. The objective of this study was to purify and partially characterize α_1-PI from the serum of the common marmoset (*Callithrix jacchus*). The purified marmoset α_1-PI will then be utilized for the development of an immunoassay as a noninvasive marker of chronic lymphocytic enteritis and protein loss in marmosets.

2.2 **Materials and methods**

2.2.1 Marmoset serum

Surplus serum submitted to the Gastrointestinal Laboratory for routine testing (NEPRC), and sera harvested from euthanized common marmosets as part of routine colony management procedures (SNPRC) were used. These procedures were approved by the animal care and use committee at the respective institution. Serum samples were stored at -80^{o}C and shipped on dry ice.

2.2.2 Affinity column chromatography

An empty column (XK 16/20, GE Healthcare Life Sciences, Piscataway, NJ) was packed with 11.8 ml of alpha$_1$-antitrypsin select resin (alpha$_1$-antitrypsin select media, GE Healthcare Biosciences, Piscataway, NJ) as per manufacturer's instructions. This column was stored in 20% ethanol at +4^{o}C until further use. Pooled marmoset serum was diluted in a 1:10 dilution with Buffer

A; [20 mM Tris(hydroxymethyl)aminomethane hydrochloride (Sigma-Aldrich, St. Louis, MO), 50 mM sodium chloride (Sigma-Aldrich, St. Louis, MO), pH 7.4]. After thorough mixing, the sample was filtered through a 0.2 µm pore size filter and applied to the column coupled with a preparative fast protein liquid chromatography (FPLC) system (ÄKTA, GE Healthcare Biosciences, Piscataway, NJ). Before loading the serum sample, the column was equilibrated with 50 ml of Buffer A at a flow rate of 2 ml/min, and then with 50 ml of Buffer B; [20 mM Tris-HCl, 2 M magnesium chloride hexahydrate (Sigma-Aldrich, St. Louis, MO), pH 7.4] at 4 ml/min. The sample was injected and the absorbance at 280 nm monitored. The peak that resulted from the injection was allowed to return to baseline. Subsequently, buffer B was applied at a flow rate of 4 ml/min. One milliliter fractions were collected during the elution phase and analyzed using sodium dodecyl sulphate electrophoresis polyacrylamide gel electrophoresis (SDS–PAGE) with precast gels under reducing conditions (NuPAGE® Novex® 10% Bis-Tris mini-gels, Life Technologies, Grand Island, NY) and using a known molecular weight standard marker (Mark 12, Life Technologies, Grand Island, NY). The affinity column was cleaned using 50 ml of each: 2 M sodium chloride (Sigma-Aldrich, St. Louis, MO), 6 M guanidine HCl (Sigma-Aldrich, St. Louis, MO), and 10% isopropyl alcohol (Sigma-Aldrich, St. Louis, MO) in succession, and then stored in 20% ethanol (Decon Labs, King of Prussia, PA). All the buffers used were prepared in ultrapure water with 18.2 MΩ.cm resistivity at +25°C (Milli-Q, EMD Millipore Corporation, Billerica, MA), and were filtered (0.2 µm), and degassed before use.

2.2.3 Buffer exchange and concentration

Eluted fractions that contained protein of the expected molecular weight of marmoset α_1-PI (approximately 50 kDa) were pooled. Buffer was exchanged to buffer C [10 mM sodium

phosphate (Sigma-Aldrich, St. Louis, MO), pH 6.95] using a dialysis cassette (Slide-A-Lyzer dialysis cassettes, 10K MWCO, Thermo Fisher Scientific, Rockford, IL) as per manufacturer's recommendations. The dialyzed material was concentrated to a final volume of 4 ml using centrifugal filters (Amicon® Ultra 15 ml filter, EMD Millipore Corporation, Billerica, MA) and stored at -80^{o}C.

2.2.4 Ceramic hydroxyapatite chromatography (CHT)

A 5 ml pre-packed ceramic hydroxyapatite chromatography type II media cartridge (Bio ScaleTM Mini CHT Type II, Bio-Rad, Hercules, CA) was used. The CHT column was connected to the purification system and prepared for injection after equilibration with 25 ml of buffer C, followed by 25 ml of buffer D [400 mM sodium phosphate (Sigma-Aldrich, St. Louis, MO)**,** pH 6.95]. The concentrated protein was injected onto the CHT column at a flow rate of 1 ml/min and the flow-through was collected in 1 ml fractions. These fractions were screened for protein activity using the trypsin inhibitory activity assay (see 2.7.1). Fractions that had the highest activity were pooled, buffer exchanged as before to phosphate buffered saline pH 7.2 (BupH phosphate buffered saline packs, Thermo Fisher Scientific Inc., Rockford, IL), and concentrated to a final protein concentration of 1 mg/ml. The concentrated protein was stored at –80°C for further characterization.

2.2.5 Gel electrophoresis and purity

Protein purity was determined by reducing SDS-PAGE using precast 10% Bis-Tris mini-gels (NuPAGE® Novex® 10% Bis-Tris mini-gels, Life Technologies, Grand Island, NY) and subsequent staining with a ready-to-use colorimetric stain formulated with Coomassie dye R-250

(Imperial protein stain, Thermo Fisher Scientific, Rockford, IL) as per manufacturer's recommendation.

2.2.6 Protein concentration

Protein concentration was determined using the Bradford protein assay (Thermo Scientific Pierce, Rockford, IL).

2.2.7 Proteinase inhibitory activity

2.2.7.1 Trypsin inhibitory activity

Trypsin inhibitory activity was measured using a previously established assay[44] and was used to monitor the purification process. Briefly, bovine trypsin (Sigma-Aldrich, St. Louis, MO) and Nα-benzoyl-DL-arginine-*p* nitroanilide (BAPNA, Sigma-Aldrich, St. Louis, MO) were used as the proteinase and the substrate, respectively. The change in absorbance due to the release of p-nitroanilide was used to measure trypsin activity in a 96-well microtitre plate. The activity was measured over a 15 minute interval at a wavelength of 405 nm on a kinetic plate reader. Absorbance of each well was measured every 30 seconds. The maximum rate of change in absorbance was automatically calculated by integrating across the 30 different measurement points and was used for calculating trypsin activity. For the purpose of this study, one arbitrary unit of specific activity was defined as the amount of marmoset α_1-PI necessary to reduce the maximum rate of change of absorbance of the test wells to 50% of the negative control well.

2.2.7.2 Elastase inhibitory activity

Elastase inhibitory activity was assayed as described previously.[148] Briefly, methoxysuccinyl-Ala-Ala-Pro-Val-p-nitroanilide was used as the substrate for human neutrophil elastase (SERVA Electrophoresis GmbH, Heidelberg, Germany) in a 96-microwell format. Elastase inhibition was determined by the absence of an increase in the absorbance, measured over 15 minutes at a wavelength of 405 nm in a microwell where marmoset α_1-PI was pre-incubated with the enzyme.

2.2.7.3 Chymotrypsin inhibitory activity

Chymotrypsin inhibitory activity was assayed as described previously.[106] Briefly, inhibitory activity was demonstrated using 0.35 mM succinyl-Ala-Ala-Pro-Phe-p-nitroanilide (SAPNA, Sigma-Aldrich, St. Louis, MO) as substrate with a chymotrypsin solution of 15 U/ml in a microtitre 96 well plate and reading the activity using a plate reader at 405 nm. Similar to other enzyme inhibitory activities, when the enzyme was pre-incubated with marmoset α_1-PI, the lack of increase in the absorbance measured over 15 minutes at a wavelength of 405 nm was used to determine chymotrypsin inhibitory activity.

2.2.8 Determination of molecular weight and relative molecular mass

Molecular weight was estimated by using 10% Bis Tris polyacrylamide gel electrophoresis under reducing conditions, against a standard protein ladder (Mark 12, Life Technologies, Grand Island, NY). The molecular weight was estimated using gel analysis software (Quantity One 1-D Analysis Software, Bio-Rad Laboratories, Hercules, CA). The relative molecular mass (Mr) was estimated using surface-enhanced laser desorption/ionization time of flight mass spectrometry

(SELDI-TOF-MS;Protein Chip® SELDI, System, Bio-Rad Laboratories, Hercules, CA) using 6 ng of purified marmoset α_1-PI immobilized onto a nonselective normal phase chromatographic array (NP 20 Protein Chip® array, Hercules, CA).

2.2.9 Isoelectric point

The pI (isoelectric point) was estimated using native isoelectric focusing with a linear pH gradient from 3 to 10 in a vertical format on a precast polyacrylamide gel (Novex® pH 3-10 IEF protein gel, Life Technologies, Grand Island, NY).

2.2.10 Specific absorbance

Specific absorbance of marmoset α_1-PI was determined by using the absorbance as measured by the spectrophotometer (NanoDrop 1000, NanoDrop products, Wilmington, DE) and the corresponding protein concentration as determined by a Bradford protein assay.

2.2.11 N-terminal amino acid sequence and tryptic peptide mass fingerprint (PMF)

The purified protein was submitted to the Protein Chemistry Laboratory (Department of Biochemistry and Biophysics, Texas A&M University, TX) for N-terminal amino acid sequencing using automated Edman's protein sequencing on a Model 492 automated protein sequencer (Applied Biosystems, Foster City, CA). This was followed by comparing the sequence against an established database. Homology between species was determined using the percentage of homologue amino acids of the amino acid sequence portion determined.

The purified protein on a gel was submitted to the Protein Chemistry Laboratory at Texas A&M University for tryptic mass fingerprinting. Briefly, the gel was subjected to tryptic digestion

and the resulting peptides were extracted and the unfractionated mixture was analyzed by Matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF-MS). Tandem mass spectra were extracted, charge state deconvoluted and deisotoped by Mascot Distiller version 2.2.1. All MS/MS samples were analyzed using Mascot (Matrix Science, London, UK; version Mascot) and X! Tandem (The GPM, thegpm.org; version CYCLONE (2010.12.01.1)). The spectrum modeler, X! Tandem was set up to search a subset of the NCBInr_20110312 database. Mascot was set up to search the NCBInr_20110312 database (unknown version, 13366630 entries) assuming the digestion with trypsin. Mascot and X! Tandem were searched with a fragment ion mass tolerance of 0.80 Da and a parent ion tolerance of 1.5 Da. Oxidation of methionine and iodoacetamide derivative of cysteine were specified in Mascot and X! Tandem as variable modifications. Scaffold (version Scaffold_3.1.2, Proteome Software Inc., Portland, OR) was used to validate MS/MS based peptide and protein identifications. Peptide and protein identifications were accepted as per specifications from the Peptide Prophet algorithm[74] and Protein Prophet algorithm.[109]

2.2.12 Immunologic cross-reactivity and Western blotting

Polyclonal antibodies were raised in a New Zealand white rabbit by inoculation with 200 μg of purified marmoset α_1-PI emulsified in Freund's complete, and then by repeated injections of 100 μg of purified marmoset α_1-PI in Freund's incomplete adjuvant by a commercial antibody production service (Lampire Biological Laboratories, Pipersville, PA). Specificity of antibodies raised were determined by using a Western blot, using marmoset serum, pooled protein fractions from the A_1AT select affinity column (Alpha$_1$-antitrypsin select medium consists of binding ligands made from camelidae-derived single domain antibody fragments, obtained after

immunization of lamas with human A_1AT protein on an agarose matrix), and purified marmoset α_1-PI. A 1:5000 dilution of the primary antibody with approximately a titer of 80%, and a 1:50,000 dilution of secondary antibody (Goat anti-Rabbit IgG, Pierce, Thermo Fisher Scientific Inc., Rockford, IL) were used. Cross-reactivity of sera raised against marmoset α_1-PI was assayed using the radial double immunodiffusion against human, dog, cat, mouse, and rat serum. Various non-human primate sera from the Houston zoo and the Phoenix zoo were also tested for cross-reactivity with marmoset α_1-PI. The primate species tested included the Geoffrey's marmoset, pied tamarin, cotton topped tamarin, golden lion tamarin, ring tailed lemur, black and white ruffled lemur, red fronted lemur, mandrill, red capped mangabey, Allen's swamp monkey, De Brazza's monkey, Schmidt's monkey, orangutan, Rhesus monkey, pigtail monkey, and the chimpanzee. Marmoset serum was used as a positive control and phosphate buffered saline as negative control.

2.3 Results

2.3.1 Purification of marmoset α_1-PI

Marmoset α_1-PI was successfully purified from marmoset serum. Results from an exemplary purification are summarized in Table 1. Progression of the purification process was monitored by use of SDS-PAGE and the trypsin inhibition assay. Marmoset α_1-PI was retained on the A_1AT select affinity column. The majority of other serum proteins were observed in the flow through (Figure. 1). Retained marmoset α_1-PI and other proteins were eluted with Buffer B. The eluted fractions were visualized on a gel, and fractions with marmoset α_1-PI were pooled and concentrated. Proteolytic activity could not be measured during this phase because of the interference from magnesium ions present in the buffer. Marmoset α_1-PI was then concentrated, buffer exchanged, and loaded onto the ceramic hydroxyapatite (CHT) column. Marmoset α_1-PI

did not bind to the column and this step helped to remove other major protein contaminants (Figure. 2). The overall yield of the purification protocol was about 11.9%. There was a 19-fold increase in the purification. After the final concentration step marmoset α_1-PI was visualized as a single band on SDS-PAGE after staining (Figure. 3).

Table 1. Sequential purification of marmoset α_1-PI from 2 ml of marmoset serum. Reprinted with permission.[120]

Purification stage	Protein content (mg)	Total inhibitory activity (units)	Specific activity (U/mg of protein)	Recovery (%)	Purification (fold)
Serum	111.57	250	2.2	100	1
Affinity chromatography	2.0	59	29.5	23.6	13
Ceramic hydroxyapatite chromatography	0.68	30	43.7	11.9	19

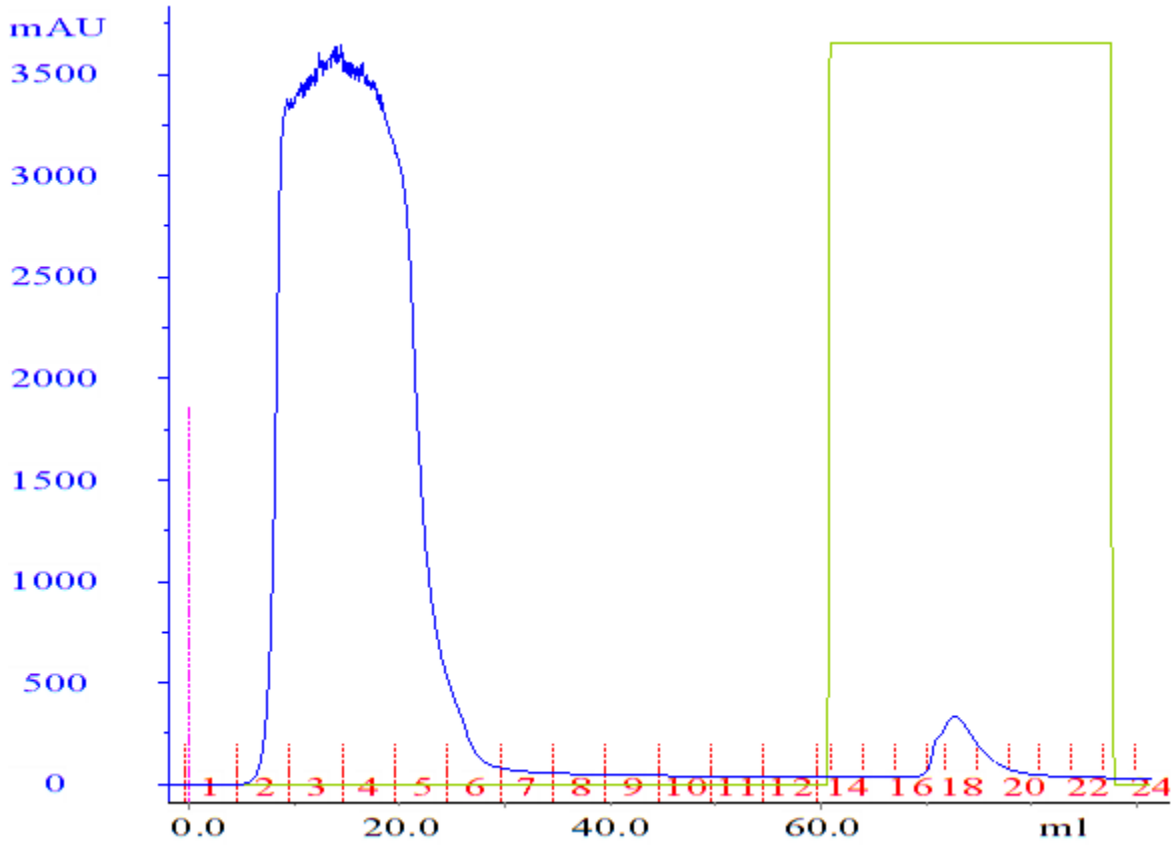

Figure. 1. Chromatogram for purification with the A_1AT select immunoaffinity column. Diluted marmoset serum was applied onto the column and the column washed with buffer A. Bound marmoset α_1-PI was eluted by use of 100% buffer B. Fractions in tubes 17, 18 and 19 on the chromatogram (third peak) showed the area corresponding to the band with the highest concentration of marmoset α_1-proteinase inhibitor. Protein elution was monitored by measurement of absorbance (in milli-absorbance units [mAU]) at 280 nm. Reprinted with permission.[120]

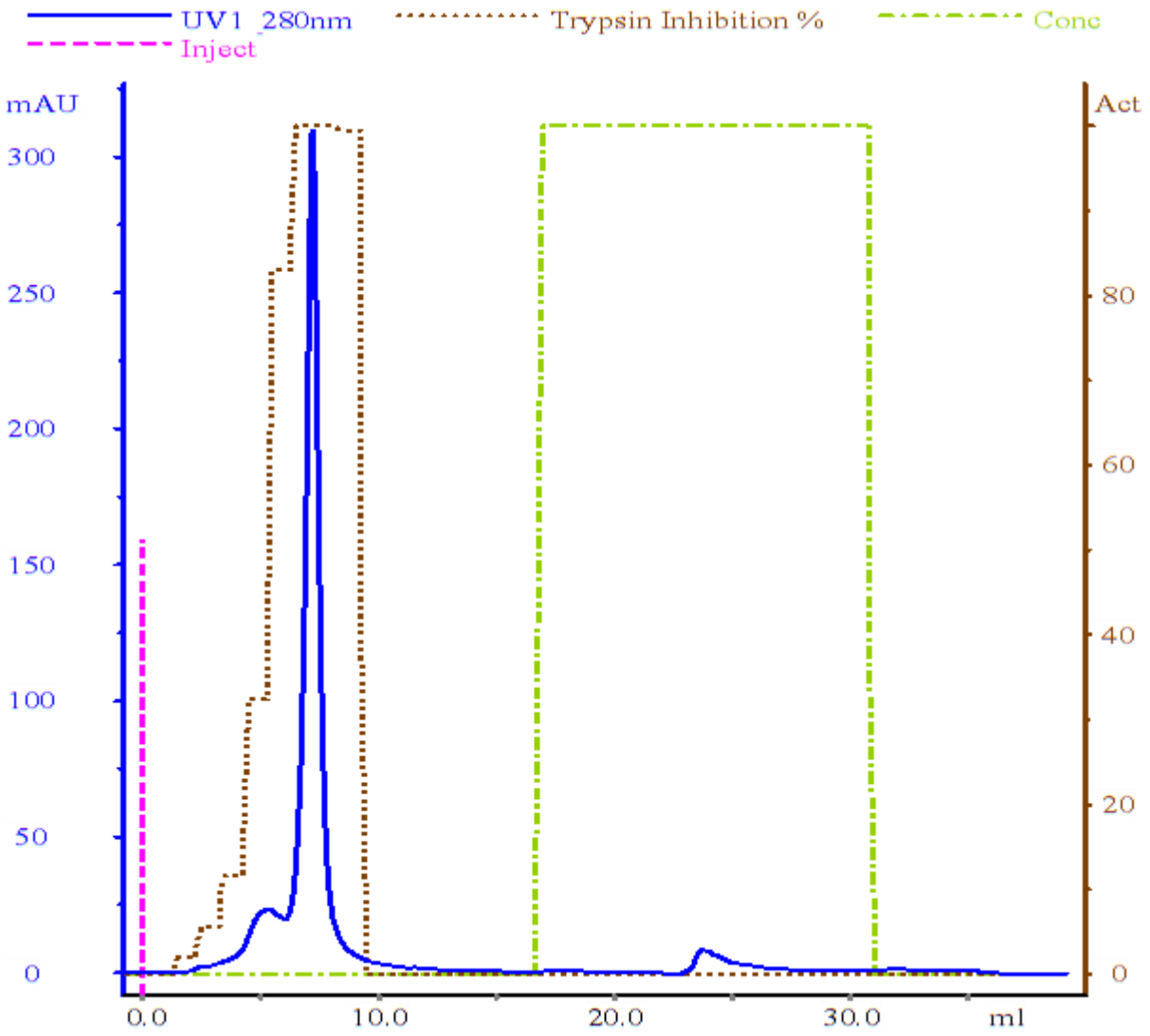

Figure. 2. Ceramic hydroxyapatite chromatography of partially purified marmoset α_1-PI. Buffer C, at a flow rate of 1 ml/min, was used as the mobile phase. Fractions of the second peak showed the highest trypsin inhibition of 100% and were pooled and concentrated. Protein elution was monitored by absorbance (in milli-absorbance units [mAU]) at 280 nm. Reprinted with permission [120]

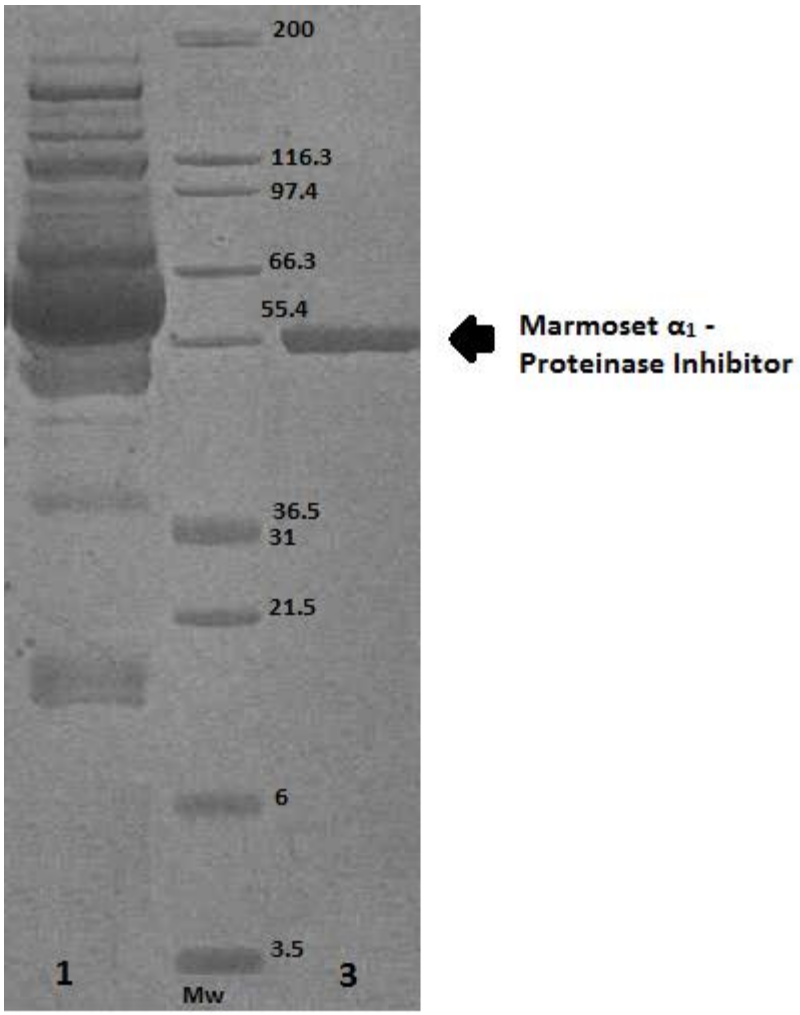

Figure. 3. Imperial protein stain stained SDS-PAGE of partially purified marmoset α_1-PI. The molecular weights of a set of molecular mass markers (Mw) are shown in middle of the gel. The following material was loaded into the lanes: lane 1: marmoset serum, 3: pure α_1-PI after ceramic hydroxyapatite chromatography. Reprinted with permission.[120]

2.3.2 Characterization of marmoset α_1-PI

The estimated molecular mass on a 10% Bis Tris polyacrylamide electrophoresis gel was about 54 kDA. The relative molecular mass (M_r) was estimated 51,677. The pI (isoelectric point) of marmoset α_1-PI was revealed by 6 bands in close proximity between 4.8 and 5.4. The specific absorbance of marmoset α_1-PI was 1.6. The N-terminal amino acid sequence of the last 16 amino acids were EDPQGDAAQKMDTSHH, using the single letter code. Homology between the amino acid sequences of marmoset α_1-PI and other species like human, dog, cat, horse and sheep were 81%, 50%, 44%, 44%, and 38%, respectively (Figure. 4). This sequence yielded two hits on UNIPROTKB (http://www.uniprot.org) for the common marmoset; one was an unidentified protein (F7GL69_CALJA) and the other alpha$_1$-antitrypsin (U3FMP8_CALJA) with 100% identity. The tryptic peptide mass fingerprint (PMF) and database search of the PMF data showed sequence similarity with alpha$_1$-proteinase inhibitor. Immunologic cross-reactivity determined using hyperimmune serum raised against marmoset α_1-PI was observed only with serum from the Geoffrey's marmoset among all of the species tested.

2.4 Discussion

No cross-reactivity was observed between marmoset samples on human and canine assays and purification of marmoset α_1-PI was needed to develop a suitable fecal immunoassay. In this study, we report the successful purification of α_1-PI from marmoset serum using a two-step chromatographic procedure. The identity of marmoset α_1-PI was confirmed by tryptic peptide mass fingerprinting, primary sequence determination of the N-terminus of the first 16 amino acids, and by enzyme inhibition assays.

Species	N-terminal amino acid sequence																Homology (%)
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	
Marmoset	E	D	P	Q	G	D	A	A	Q	K	M	D	T	S	H	H	
Human	E	D	P	Q	G	D	A	**V**	Q	**E**	**T**	D	T	S	H	H	81
Dog	E	**G**	**L**	Q	G	D	A	**V**	Q	**E**	**T**	D	D	**P**	**E**	H	50
Cat	E	**G**	**L**	Q	G	**A**	A	**V**	Q	**E**	**T**	**V**	**A**	S	**Q**	H	44
Horse	E	D	**L**	Q	G	**X**	A	**V**	Q	**E**	**T**	D	**A**	**D**	**K**	**D**	44
Sheep	**Q**	**V**	**L**	Q	G	**H**	A	**V**	Q	**E**	**T**	D	**D**	**T**	**A**	H	38

Figure. 4. N-terminal amino acid sequences of α_1-PI from different species. This table shows the N-terminal amino acid sequence of the last 16 amino acid residues of α_1-PI in the common marmoset, cat, dog, human being, sheep, and horse. Amino acid residues in bold and underlined indicate differences in the sequence, with respect to marmoset α_1-PI.

The described protocol is quick, reproducible, and has a good yield. Purified marmoset α_1-PI was sufficiently pure as indicated by a single band on SDS-PAGE. Marmoset α_1-PI purification has not been reported elsewhere in literature. Serum availability for purification is a major limitation in marmosets because of their small body size and thus limited blood volume; hence a process with a high yield was necessary for purification. Though a variety of methods have been employed in the purification of serum alpha$_1$-proteinase inhibitors from other species, the use of immunoaffinity chromatography has only been described for human sera. Alpha$_1$-antitrypsin (A_1AT) select medium consists of A_1AT binding ligands attached to an agarose matrix. A_1AT binding ligands are made of camelidae-derived single domain antibody fragments, which were obtained from lamas after immunization with human A_1AT protein. Very recently, using the same immunoaffinity media, human alpha$_1$-antitryspin select was purified with a 60% yield in the initial step, with an overall yield of 42% for the entire purification protocol.[174] Our yield was lower at ~12%. This difference may be attributable to the difference in affinity of marmoset α_1-PI for the A_1AT select resin. During the purification process, we observed that after repeated usage of the A_1AT Select column, a thorough multistep cleaning in-place protocol was necessary to maintain column performance. Trypsin inhibitor assay was also found to be altered by high magnesium concentrations in the elution buffers during the immunoaffinity purification, thus a buffer exchange was necessary.

The characteristics of marmoset α_1-PI are similar to those of α_1-PI from other species. The pI between 4.8-5.4 is close to that reported in the cat (4.3-4.7),[44] rat (4.3-4.8),[83] guinea pig (4.5-4.9),[151] rabbit (4.8-5.0),[79] human (4.55-4.47),[116] dog (4.7-4.9),[97] and sheep (4.95).[102] Purified marmoset α_1-PI did retain its ability to inhibit the proteolytic activity of bovine trypsin, chymotrypsin, and human elastase. Similar enzyme inhibitory activity has been reported for α_1-PI purified from sheep.[55] The observed molecular mass of marmoset α_1-PI is similar to that reported for other species [range: 47 – 72 kDa].[17,44,97,116,129] The protein identity was confirmed by N-terminus amino acid sequencing and PMF which are widely accepted methods for protein identification.[51] A homology of 81.3% was observed between human and marmoset α_1-PI. No immunological cross reactivity was observed across other species tested, except for the Geoffrey's marmoset. This limited cross-reactivity was expected and has been reported earlier.[129]

In summary, this is the first report of the successful purification and partial characterization of marmoset alpha$_1$-proteinase inhibitor. The purified protein showed considerable homology to alpha$_1$-proteinase in other species with regard to activity, M_r, and pI. The purification of the protein and development of the hyperimmune serum described here would serve as a prelude to the development of an immunoassay for the measurement of fecal marmoset alpha$_1$-proteinase inhibitor concentrations.

CHAPTER III

DEVELOPMENT AND ANALYTICAL VALIDATION OF A RADIOIMMUNOASSAY FOR THE QUANTIFICATION OF ALPHA$_1$-PROTEINASE INHIBITOR IN SERUM AND FECES FROM THE COMMON MARMOSET (*CALLITHRIX JACCHUS*)

3.1 Introduction

The common marmoset (*Callithrix jacchus*) is a New World primate that is used in biomedical research.[90] The popularity of the marmoset is related to its small size, relatively easy maintenance, absence of certain zoonotic diseases, multiple births at each parturition, and relatively short life span.[1] However, inflammation of the gastrointestinal tract has been commonly reported in common marmosets kept in captivity. The disease has been deemed to be endemic, with the reported prevalence ranging from 28–60 % in the laboratory setting.[88] Chronic lymphocytic enteritis (CLE) is associated with chronic progressive weight loss in adults despite a good appetite, failure to thrive in juveniles. Diarrhea may or may not be present. The only consistent clinical pathological abnormalities are a mild anemia and hypoproteinemia.[11,88] The disease is often terminal with affected marmosets either dying of being euthanized. The current gold standard for diagnosis is necropsy.

The development of a non-invasive test that may allow early ante-mortem diagnosis would facilitate screening and removal of affected marmosets before they are enrolled in research trials or breeding programs, thereby reducing the impact of this disease. Given that hypoproteinemia, particularly hypoalbuminemia, has been reported consistently in marmosets with chronic inflammation of the intestinal tract, we hypothesize this hypoproteinemia may be due an intestinal loss of protein as seen in other species with inflammatory bowel disease. The

gold standard for detecting gastrointestinal protein loss is the excretion of ^{51}Cr-albumin in feces, which is not feasible as a routine diagnostic test given the radiation hazards involved.

Alpha$_1$-proteinase inhibitor (α_1-PI) is a serum protein produced by the liver that is only lost in minimal quantities into the GI tract during health. Given the similar molecular weight to albumin, it is lost into the intestinal tract at a similar rate as is albumin. However, unlike albumin, it is resistant to degradation by digestive enzymes and bacteria within the intestinal lumen. Intact fecal α_1-PI can be detected by immunoassays and have been employed to detect increased enteric protein loss in humans,[22] dogs,[96] and cats.[26] In dogs with protein losing enteropathy, increased fecal α_1-PI concentrations are thought to precede hypoalbuminemia. Assays are species specific and do not cross react.[146] We described the purification and partial characterization of marmoset α_1-PI from marmoset serum.[120]

The aims of this study were to develop and analytically validate an RIA for the quantification of α_1-PI in serum samples and fecal extracts from marmosets and to establish reference intervals for serum and fecal α_1-PI concentrations in healthy marmosets.

3.2 Materials and methods

3.2.1 Marmoset serum & fecal samples

Serum and fecal samples previously submitted to the Gastrointestinal Laboratory for research testing, and serum harvested from common marmosets euthanized as part of routine colony management procedures stored at -80^{o}C and shipped on dry ice were used for assay development and analytical validation.

3.2.2 Animals

A total of 30 marmosets from two research colonies of common marmosets accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (Southwest National Primate Research Center, Texas Biomedical Research Institute, San 307 Antonio, Texas and the Barshop Institute for Longevity & Aging Studies University of Texas Health Science Center at San Antonio) were used to establish reference intervals for serum and fecal α_1-PI concentrations in healthy marmosets. These procedures were approved by the animal care and use committee at the respective institutions (AUP 1259CJ & AUP 06120X).

Paired serum and naturally passed fecal samples were collected from each animal. Since it has been demonstrated that fecal α_1-PI concentrations can have day-to-day variations in dogs, and no data exists for the common marmoset, fecal samples were collected for three consecutive days from each animal. Fecal samples were collected as soon after defecation as possible in pre-weighed polypropylene tubes, and stored at -80^{o}C until they were shipped on dry ice.

3.2.3 Extraction of fecal samples

Fecal samples were thawed and then the extraction was done using phosphate buffered saline supplemented with 5% newborn calf serum, 1% Triton X-100, and 0.25 mM thimerosal in a 1:5 dilution using approximately 1.0 g wet weight of feces. The fecal samples were homogenized by vortexing for 20 minutes, then centrifuged for 20 min at 2,100 x g and 5°C, and then the supernatants [fecal extract] were collected using serum filters, and were stored frozen at –80°C until analysis. This extraction is similar to the extraction described for the canine assay.[59]

3.2.4 Purification of marmoset α_1-PI

Marmoset alpha$_1$-proteinase inhibitor was purified from marmoset serum by affinity chromatography, and ceramic hydroxyapatite chromatography as described previously.[120]

3.2.5 Antibody production

Polyclonal antibodies were raised in New Zealand white rabbits using a commercial antibody production service (Express-Line PLUS protocol; Lampire Biological Laboratories, Pipersville, Pennsylvania, USA). Purified marmoset α_1-PI in phosphate buffered saline was used as the antigen and antiserum specificity was tested as previously described.[120] Antiserum obtained after the fourth booster injection was selected for the RIA procedure with a final dilution, of 1:5,000.

3.2.6 Dilution of serum samples

Serum samples were diluted 1:32,000 in radioimmunoassay buffer (RIAB, 0.05 M sodium phosphate, with 0.02% (w/v) sodium azide and 0.5% (w/v) bovine serum albumin (pH 7.5)) for the RIA.

3.2.7 Dilution of fecal samples

Previously stored fecal extracts were thawed and diluted 1:200 in RIAB for the RIA.

3.2.8 Tracer radioiodination

The tracer was produced by iodination of pure marmoset α_1-PI with ^{125}I, using the chloramine T method.[62] A stir bar [8 mm × 1.5 mm; VWR Scientific, West Chester, Pennsylvania,

USA] was placed in a polypropylene test tube (75 mm × 12 mm; VWR Scientific) and 10 µL of 0.25 M sodium phosphate buffer [Sigma-Aldrich, St. Louis, MO] was pipetted into it, which was then placed over a stir plate. With the use of a Hamilton syringe (VWR Scientific) 10 µL of carrier free I^{125} [NaI, 1.0 mCi at time of production] (Perkin Elmer, Waltham, MA) was dispensed into the tube. Then the following solutions were added in succession: 10 µg of the marmoset α_1-PI, 10 µL of the chloramine T solution [2 mg/mL], 100 µL of a sodium metabisulfite solution (0.4 mg/mL), and 860 µL of a potassium iodide solution (2 mg/mL). All the solutions used were dissolved in 0.05 M sodium phosphate (pH, 7.5). The iodinated mα_1-PI fraction was separated from the free iodide by size-exclusion chromatography on a disposable column equilibrated with 10% (wt/vol) bovine serum albumin (PD-10; GE Healthcare, Piscataway, New Jersey, USA) following the manufacturer's directions. The RIA buffer (RIAB) was used as the mobile phase, and 1-mL fractions were collected. The fraction containing the peak protein concentration was used as the tracer. The tracer was adjusted with RIA buffer to approximately 45,000 counts/minute/100 µL tracer and kept at 4°C until further use.

3.2.9 Radioimmunoassay procedure

All polypropylene reaction tubes (VWR Scientific) were set up in a duplicate fashion. The tubes were labelled total count (TC), nonspecific binding (NB), reference blank (B0), and standards labelled 100, 50, 20, 10, 5, 2, and 1 ng/mL. The tubes were filled accordingly: TC received 100 µL tracer only, NB received 100 µL tracer and 200 µL RIAB, B0 received 100 µL tracer, 100 µL antibody solution, and 100 µL RIAB. The standards received 100 µL tracer, 100 µL antibody solution, and 100 µL standard solution of 100, 50, 20, 10, 5, 2, and 1 ng/mL mα_1-PI in RIAB, respectively. Unknown samples received 100 µL tracer, 100 µL antibody solution, and 100

μL of the unknown sample. Tubes were vortexed and then incubated for 4 hours at room temperature. At the end of the incubation period, all, except the tubes labeled TC, received 100 μL of a rabbit carrier serum (1 mL normal rabbit serum mixed with 99 mL RIAB) and 1 mL of a commercially available precipitation solution (N6; Siemens Healthcare Diagnostics Inc., Tarrytown, New York, USA). All tubes except the TC tubes were vortexed and then centrifuged at 3,360 x g and 10°C for 30 min. The supernatant of all tubes, were carefully decanted. The tubes were visually inspected to ensure that the pellets were present. The tubes were placed in a gamma counter (Riastar; Packard Instrument Company, Meriden, Connecticut, USA) and counted for 1 minute. A standard curve was generated using a log/logit curve fit. Mα_1-PI concentrations were marked on the x-axis in a logarithmic fashion and values on the y-axis were calculated from $y = \log_e((B_{standard}/B_0)/(1 - (B_{standard}/B_0))$ with B standard being the counts per minute for each standard and B0 being the counts per minute for the reference blank. Test samples containing mα_1-PI in concentrations greater than the working range of the assay were further diluted by a factor of 2 and re-assayed.

3.2.10 Radioimmunoassay validation

The mα_1-PI RIA was analytically validated by determining the lower limit of the assay, the linearity, accuracy, precision, and reproducibility by evaluating assay sensitivity, dilutional parallelism, spiking recovery, intra-assay variability, and inter-assay variability. Serum and fecal samples were selected at random from the available samples and efforts were made to ensure that they fell into different areas of the working range of the assay. Serum samples used for validation were generated from a pool of several samples because marmosets are significantly smaller than companion animals and serum volumes greater that 0.5 mL were not available for most animals.

The lower limit of the assay was determined by analyzing 10 duplicates of the B0 tubes and calculating the mean and the standard deviation of the raw counts for the zero mα1-PI concentration. The mα1-PI concentration that corresponded to the mean count minus three standard deviations was estimated on the standard curve and defined as analytic sensitivity (lower limit of the working range) of the RIA. The upper limit of the working range was taken as the highest standard. Linearity of the assay was determined by use of 8 serum samples in two fold dilutions from 1:32,000 to 1:512,000, and 4 fecal samples in two fold dilutions from 1:1 to 1:16. Accuracy of the assay was tested by spiking 4 sera and 4 fecal extracts with known concentrations of pure mα1-PI (20, 50, and 100 µg/L for serum samples and 20, 50, and 100 µg/g for fecal extracts). Recovery was determined by calculating the ratio of the observed/expected X 100. Intra-assay variability or precision of the assay was evaluated by assaying 8 serum samples, and 8 fecal extracts 4 times within the same assay, followed by calculating the coefficient of variation: CV % = (SD/mean) X 100. Inter-assay variability or reproducibility of the assay was determined by analyzing 8 sera and 8 fecal extracts in 3 consecutive assays and calculating inter-assay coefficient of variation; CV % = (SD/mean) X 100. To evaluate if there was a position effect within the assay (end of-run effect) two samples were tested in the same run by analyzing each sample 24 times in the same assay, with 8 duplicates placed in the beginning, middle, and at the end of the same assay. Mean mα1-PI concentration calculated from these positions on the assay were compared using a Kruskal-Wallis test (rank sum) test.

3.2.11 Reference interval determination

The reference interval for serum concentrations of marmoset α1-PI were determined by calculating the central 95th percentile of serum concentrations measured in the 30 healthy

marmosets from both the colonies. Fecal α_1-PI concentrations were measured in the same 30 marmosets, and the reference interval was established calculating the 95th central percentiles of both, the mean and the maximum fecal α_1-PI concentrations in the fecal samples from 3 consecutive days. The coefficient of variation was also calculated between the three separate fecal samples collected. None of the marmosets had any clinical signs of wasting or lymphocytic enteritis and therefore at the time of collection of serum or feces and were deemed to be healthy.

3.2.12 Statistical analysis

Statistical analyses were performed using a statistical software program [Graph Pad prism software, GraphPad Software, Inc. La Jolla, CA 92037 USA) and statistical significance was set at $p < 0.05$.

3.3 Results

The lower limit of the assay was calculated to be 0.8 µg/L. The upper limit of the working range was calculated to be 100.6 µg/L. Eight serum samples in two fold dilutions from 1:32,000 to 1:512,000 and 4 fecal samples in two fold dilutions from 1:1 to 1:16 were assayed. The observed to expected ratios (O/E) for serial dilutions were (minimum-maximum (mean ± SD)) 89.9 – 123.0 % (106.0 ± 11.5%) for serum, and 90.6 – 132.7% (107.6 ± 19.2%) for fecal extracts [Table 2, 3]. The O/E for spiking recovery for 3 different spiking concentrations were (minimum-maximum (mean ± SD)) 97.6 – 104.4% (101.3 ± 3%) for serum and 97.5 – 101.4% (99.2 ± 1.8%) for serum [Table 4, 5.]. Intra-assay coefficients of variation (CV %) (n=8) were 1.7, 2.8, 2.8, 3.5, 4.6, 4.6, 5.6, and 10.6% for serum samples and 2.2, 2.3, 2.7, 3.1, 4.0, 4.3, 4.3, and 5.1% for fecal extracts (n=8) [Table 6, 7]. Also, inter-assay coefficients of variation (CV %) (n=8) were 1.3, 5.2, 5.6, 6.3,

8.0, 8.2, 8.4, and 9.9% for serum samples and were 1.0, 1.1, 2.6, 3.2, 3.3, 3.8, 4.8, and 6.7% for fecal extracts (n=8) [Table 8, 9]. No significant differences in mean α_1-PI concentrations depending on position of samples in the assay were observed (p=0.4128).

Table 2. Dilutional parallelism of a RIA for the measurement of serum α_1-PI concentrations in the common marmoset

Serum Sample ID	Dilution	Observed α_1-PI Concentration (µg/L)	Expected α_1-PI Concentration (µg/L)	O/E %	Average O/E %
S1	1:32000	28.7	N/A	N/A	
	1:64000	16.2	14.3	113.3	
	1:128000	7.1	7.2	98.8	
	1:256000	4	3.6	111.6	
	1:512000	1.4	1.8	76.1	99.9
S2	1:32000	31.4	N/A	N/A	
	1:64000	19.1	15.7	121.5	
	1:128000	7.7	7.9	98.6	
	1:256000	5.7	3.9	144.9	
	1:512000	2.1	2	109.3	118.6
S3	1:32000	29	N/A	N/A	
	1:64000	17.7	14.5	122.4	
	1:128000	7.5	7.2	103.7	
	1:256000	4.4	3.6	120.4	
	1:512000	2.6	1.8	145.5	123
S4	1:32000	30.1	N/A	N/A	
	1:64000	15.2	15.1	101.2	
	1:128000	8.7	7.5	116.2	
	1:256000	4.7	3.8	125	
	1:512000	2.2	1.9	115.4	114.5
S5	1:32000	28.2	N/A	N/A	
	1:64000	17	14.1	120.5	
	1:128000	4.6	7.1	65.8	
	1:256000	2.5	3.5	71.1	
	1:512000	1.8	1.8	102.4	89.9

Table 2. Continued

Serum Sample ID	Dilution	Observed α_1-PI Concentration (µg/L)	Expected α_1-PI Concentration (µg/L)	O/E %	Average O/E %
S6	1:32000	28.1	N/A	N/A	
	1:64000	15.2	14	108.3	
	1:128000	5.8	7	81.9	
	1:256000	3.6	3.5	103.2	
	1:512000	2.1	1.8	118.2	102.9
S7	1:32000	34.1	N/A	N/A	
	1:64000	20.3	17	118.8	
	1:128000	10	8.5	117.8	
	1:256000	3.9	4.3	90.4	
	1:512000	1.7	2.1	81.7	102.2
S8	1:32000	31.9	N/A	N/A	
	1:64000	15.4	16	96.8	
	1:128000	7.7	8	96.9	
	1:256000	3.7	4	93.5	
	1:512000	2	2	100.7	97

O/E — observed/expected

Table 3. Dilutional parallelism of a RIA for the measurement of fecal α_1-PI concentrations in the common marmoset

Sample	Dilution	Observed α_1-PI Concentration (µg/g)	Expected α_1-PI Concentration (µg/g)	Observed/ Expected %	Average O/E%
		131.2			
	1:2	61.1	65.6	93.2	
	1:4	30.8	32.8	93.8	
	1:8	15.7	16.4	95.4	
F1	1:16	7.9	8.2	96.4	94.7
		166.8			
	1:2	74.2	83.4	88.9	
	1:4	37.8	41.7	90.5	
	1:8	17.5	20.9	84.0	
F2	1:16	10.3	10.4	98.8	90.6
		90.3			
	1:2	49.1	45.1	108.8	
	1:4	27.8	22.6	123.0	
	1:8	14.9	11.3	132.3	
F3	1:16	9.4	5.6	166.6	132.7
		85.0			
	1:2	45.6	42.5	107.3	
	1:4	21.0	21.2	99.1	
	1:8	10.8	10.6	101.7	
F4	1:16	7.5	5.3	141.6	112.4

O/E — observed/expected

Table 4. Spiking recovery of a RIA for the measurement of serum α_1-PI concentrations in the common marmoset.

Sample	Spiked concentration (µg)	Observed α_1-PI Concentration (µg/g)	Expected α_1-PI Concentration (µg/g)	Observed/ Expected %	Average O/E%
	-	19.7			
	10	31.7	29.7	106.7	
	25	44.6	44.7	99.8	
S1	50	70.5	69.7	101.2	102.6
	-	21.8			
	10	35.6	31.8	111.9	
	25	46.9	46.8	100.0	
S2	50	72.6	71.8	101.1	104.4
	-	23.1			
	10	32.5	33.1	98.2	
	25	48.6	48.1	101.0	
S3	50	73.0	73.1	99.9	99.7
	-	20.5			
	10	31.0	30.5	101.6	
	25	42.3	45.5	93.2	
S4	50	69.0	70.5	98.0	97.6

O/E — observed/expected

Table 5. Spiking recovery of a RIA for the measurement of fecal α_1-PI concentrations in the common marmoset.

Sample	Spiked concentration (µg)	Observed α_1-PI Concentration (µg/g)	Expected α_1-PI Concentration (µg/g)	Observed/ Expected %	Average O/E%
	-	39.1			
	10	48.4	49.1	98.5	
	25	65.0	64.1	101.4	
F1	50	82.5	89.1	92.6	97.5
	-	52.1			
	10	66.8	62.1	107.6	
	25	79.4	77.1	102.9	
F2	50	95.7	102.1	93.7	101.4
	-	44.0			
	10	55.0	54.0	101.8	
	25	68.7	69.0	99.5	
F3	50	92.4	94.0	98.3	99.9
	-	24.0			
	10	35.7	34.0	104.9	
	25	49.5	49.0	100.9	
F4	50	65.1	74.0	88.0	97.9

O/E — observed/expected

Table 6. Precision of the RIA for serum α_1-PI concentrations in the common marmoset (intra-assay validation).

Serum Sample	Marmoset α_1-PI Concentration (µg/g)					%CV
	I	II	III	IV	Mean	
S1	-	37.2	39.7	3178.3	38.5	4.6
S2	40.0	37.4	39.7	38.1	38.8	2.8
S3	32.0	30.2	31.8	34.3	32.1	4.6
S4	32.6	33.2	34.8	34.6	33.8	2.8
S5	36.1	33.8	39.0	38.5	36.8	5.6
S6	32.3	37.9	42.6	41.8	38.6	10.6
S7	34.1	33.8	32.7	32.9	33.4	1.7
S8	30.4	31.7	32.8	30.0	31.2	3.5

CV: coefficient of variation

Table 7. Precision of the RIA for fecal α_1-PI concentration in the common marmoset (intra-assay validation)

Fecal Sample	Marmoset α_1-PI Concentration (μg/g)					%CV
	I	II	III	IV	Mean	
F1	25.4	26.5	26.9	25.7	26.2	2.3
F2	58.1	59.6	57.3	55.3	57.6	2.7
F3	40.7	41.9	43.5	44.0	42.5	3.1
F4	25.3	25.2	26.2	28.0	26.2	4.3
F5	51.6	50.5	48.9	55.0	51.5	4.3
F6	31.3	30.1	29.9	29.6	30.2	2.2
F7	12.1	11.4	12.9	12.9	12.3	5.1
F8	17.0	16.2	18.2	17.0	17.1	4.0

CV: coefficient of variation

Table 8. Reproducibility of the RIA for serum α_1-PI concentrations in the common marmoset (inter-assay validation).

Serum Samples	Marmoset α_1-PI Concentration (μg/g)				% CV
	I	II	III	Mean	
S1	40.1	38.5	42.9	40.5	5.6
S2	39.8	40.3	47.3	42.5	9.9
S3	41.5	41.2	46.0	42.9	6.3
S4	42.8	40.0	44.3	42.3	5.2
S5	40.6	41.3	40.3	40.7	1.3
S6	37.2	34.0	39.9	37.0	8.0
S7	36.7	35.9	41.7	38.1	8.2
S8	37.7	33.5	39.6	36.9	8.4

CV: coefficient of variation

Table 9. Reproducibility of the RIA for fecal α_1-PI concentrations in the common marmoset (inter-assay validation).

Fecal Samples	Marmoset α_1-PI Concentration (µg/g)				% CV
	I	II	III	Mean	
F1	60.93	60.14	61.29	60.79	0.97
F2	59.02	59.73	62.06	60.27	2.64
F3	73.85	77.42	78.57	76.61	3.21
F4	67.14	76.26	74.50	72.63	6.66
F5	39.98	42.83	42.59	41.80	3.79
F6	93.12	102.35	96.80	97.42	4.77
F7	84.19	89.94	87.20	87.11	3.30
F8	74.63	73.16	73.37	73.72	1.08

CV: coefficient of variation

A reference interval for serum and fecal α_1-proteinase inhibitor concentrations (three day mean, three day maximum) in feces was calculated using the central 95th percentile using serum and fecal samples from healthy control marmosets [Table 10]. The median coefficient of variation for fecal α_1-proteinase inhibitor concentrations between the three-day fecal samples was 21.4% (range: 2.9 – 74.3%).

3.4 Discussion

An RIA for the measurement of α_1-PI in serum samples and fecal extracts from marmosets was successfully developed. The analytical sensitivity of the assay was 0.8 µg/L. This value is adequate, considering the reference interval of 1,047 - 1,484 mg/L in serum and 32 - 124 µg/g in feces. The O/E values for the dilutional parallelism ranged from 89.9 – 123.0%, 90.6 – 132.7% and spiking recovery were 97.6 – 104.4%, 97.5 – 101.4% for serum and feces, respectively. These values indicate that the assay is linear and accurate and is close to what has been reported to be acceptable of a range of 80-120%.[157,160] Two samples, one serum sample with an O/E of 123.0% and one fecal sample with an O/E of 132.7% were outside the "acceptable" limits.

The coefficients of variability were relatively low for both intra- and inter-assay variability the maximum being 10.6%, indicating that the assay is precise and reproducible, and is well within the acceptable ranges of <15%.[157,160] No effect of position of samples on the run was detected in the assay.

The reference interval for serum marmoset α_1-PI values are comparable with the reference intervals reported for canine, feline, and human α_1-PI in plasma/serum which are 732-1,802 mg/L,[59] 250-600 mg/L,[44] and 900-1,200 mg/L,[93] respectively.

Table 10. Concentrations of serum and fecal α_1-PI in healthy marmosets (n=30)

	Concentration of marmoset α_1 - PI		
	Serum concentration (mg/L)	Three day mean fecal concentration (µg/g)	Three day maximum fecal concentration (µg/g)
Reference interval	1046 - 1484	32 -124	39 -159
Median ± SD	1225 ± 130	64 ± 27	78 ± 36
Range	983 – 1516	31-134	38-190

However, the fecal α_1-PI concentrations in healthy cats ranged from 0.04 (i.e., undetectable) to 1.72 µg/g (median: 0.51 µg/g). In adult dogs, fecal α_1-PI concentrations ranged from <2.2 to 26.8 µg/g (median: 4.7 µg/g), whereas in the 30 marmosets in this study the concentrations ranged from 30.8-134.0 µg/g (median: 63.5 µg/g). Most human fecal α_1-PI assays are performed on dried / lyophilized feces. In one study using wet weight of feces, in healthy human individuals fecal α_1-PI concentrations measure using an ELISA ranged from 1-964 µg/g and a concentration of <545 µg/g was considered normal.[22] Possible explanations for this finding are subclinical disease in some of the marmosets in this study or possibly that marmosets lose more α_1-PI into their gastrointestinal tract than carnivores.

A relatively high coefficient of variation for fecal α_1-proteinase inhibitor concentrations between the three-day fecal samples were seen in the marmosets (median 21.4%, Range 2.9 – 74.3%). The reason for this is unknown, however a similarly high CV has also been reported for dogs (median: 29.2%, range 0.0% to 102.0%).[58]

As serum and fecal samples were not readily available, stability of samples at different storage conditions were not studied. Sample collection and storage conditions used in dogs [58,107] and cats [25] were presumed to be appropriate. As the samples were collected, stored, extracted, and assayed around the same time from the two marmoset colonies, we do not expect this to affect our reference interval, however, changes in serum concentrations have been reported in dogs where samples were stored over a period of one year.[59]

A limited number of samples were available to establish the serum and fecal reference intervals for mα_1-PI. Given the small number of animals in the study, no attempts were made to make age specific reference intervals, which may have been more appropriate as younger dogs are reported to have higher concentrations of fecal α_1-PI.[58] Another limitation of this study was

that the animals that were used to establish the reference interval were presumed to be healthy based on absence of clinical signs, however, given how little is known about the disease, subclinical chronic lymphocytic enteritis cannot be ruled out as gastrointestinal biopsies were not available.

In conclusion, the RIA for the measurement of serum and fecal α_1-PI concentrations in the common marmoset described here is sensitive, linear, accurate, precise, and reproducible. Further studies are needed to determine the utility of this test for diagnosing gastrointestinal disease, particularly CLE in the marmoset.

CHAPTER IV

DEVELOPMENT AND ANALYTIC VALIDATION OF A SANDWICH ELISA FOR THE MEASUREMENT OF ALPHA$_1$-PROTEINASE INHIBITOR CONCENTRATIONS IN SERUM AND FECES FROM THE COMMON MARMOSET *(CALLITHRIX JACCHUS)****

4.1 Introduction

The common marmoset (*Callithrix jacchus*) is a New World primate that is used in biomedical research.[90] Marmosets are an attractive species for biomedical research because of their small size, ease and lower cost of maintenance, ability to be multiparous, lack of susceptibility to certain zoonotic diseases, and relatively short life span when compared to other primates.[1] However, gastrointestinal tract inflammation is commonly seen in the common marmosets colonies with the prevalence being as high as 60 % in the laboratory setting.[88] The etiology of this disease remains unknown. Clinical signs may include diarrhea, chronic progressive weight loss in adults despite a good appetite, and failure to thrive in juveniles, with anemia and hypoproteinemia. The disease is now believed to be a variant of inflammatory bowel disease, particularly when there is lymphocytic inflammation. The disease is currently known as chronic lymphocytic enteritis (CLE), however previous names included marmoset wasting syndrome[88] and bone and gastrointestinal disease of marmosets.[11] The disease leads to death, and diagnosis is currently made at necropsy.

Hypoproteinemia associated with this condition is presumed due to intestinal loss of protein. Thus, detection of enteric protein loss could facilitate screening and removal of affected

***Submitted to the American Journal of Veterinary Research (AJVR): Parambeth, JC, Lidbury J, Suchodolski J, Steiner J. Development and analytic validation of a sandwich ELISA for the measurement of alpha$_1$-proteinase inhibitor concentrations in serum and feces from the common marmoset (*Callithrix jacchus*).

marmosets thereby reducing the negative impact of this disease on research utilizing marmosets. Excretion of ^{51}Cr-albumin in feces is the test of choice for detecting enteric protein loss, but this modality is only available at a few institutions as it is associated with radiation hazards.[97] Alpha$_1$-proteinase inhibitor (α_1-PI) is a serum protein that is lost into the intestinal tract at a similar rate to albumin because it has a similar molecular weight. As it is resistant to degradation by digestive and bacterial enzymes within the intestinal lumen, fecal α_1-PI can be detected by immunoassays and has been employed to detect enteric protein loss in humans, dogs, and cats.[15,25,26,58,96] Recently, we described the purification and partial characterization of marmoset α_1-PI from marmoset serum. The aims of this study were to develop and analytically validate an enzyme-linked immunosorbent assay (ELISA) for the measurement of α_1-PI concentrations in serum samples and fecal extracts from marmosets and to establish reference intervals for serum and fecal α_1-PI concentrations in healthy marmosets.

4.2 Materials and Methods

4.2.1 Purification of α_1-PI from marmoset serum

Marmoset α_1-PI was purified from pooled marmoset serum using immunoaffinity chromatography and ceramic hydroxyapatite chromatography as previously described.[120]

4.2.2 Production and purification of marmoset α_1-PI antiserum

Polyclonal antibodies were raised in a New Zealand white rabbit by inoculation of purified marmoset α_1-PI emulsified in Freund's complete and Freund's incomplete adjuvant by a commercial antibody production service, and the specificity of antibodies were evaluated by Western blot analysis, as previously described. Rabbit anti-marmoset α_1-PI antibodies were

purified by affinity chromatography using a chromatography column (HiTrap NHS-activated HP chromatography column; GE Healthcare, Uppsala, Sweden) as per the manufacturer's instructions. The antiserum was applied to this column after lipoprotein precipitation, and buffer exchange to 75 mM Tris/HCl, 150 mM NaCl, pH 8.0, using a disposable gel filtration column (Disposable PD-10 desalting column; GE Healthcare). Purified antibodies were buffer exchanged to phosphate-buffered saline, pH 7.2, concentrated to approximately 1 mg/mL, and stored at −80°C.

One aliquot of the purified antibodies was dialyzed with 200 mM carbonate-bicarbonate, at pH 9.4, and was coupled with horseradish peroxidase using a commercially available kit (ᶜEZ-Link Plus Activated peroxidase; Thermo Scientific, Rock- ford, IL, USA) as per the manufacturer's instructions. The conjugate was purified after dialysis against 25 mM Tris/HCl, 150 mM NaCl, at pH 8.0 using a commercially available purification kit with nickel-chelated agarose (Pierce Conjugate purification kit; Thermo Scientific).

The purification was followed by another buffer exchange with 100 mM sodium phosphate, 150 mM NaCl, at pH 7.2. The resultant antibody solution was mixed with 2 parts of a commercial conjugate stabilizer solution (SuperFreeze Conjugate stabilizer; Thermo Scientific) as per manufacturer's recommendations and stored at -20^{o}C.

4.2.3 Marmoset serum & fecal samples

Serum and fecal samples submitted to the Gastrointestinal Laboratory at Texas A&M University for research testing, and sera harvested from common marmosets that were euthanized as part of routine colony management procedures, that had been stored at -80^{o}C and shipped on dry ice, were used for assay development and validation. Paired serum and naturally passed fecal samples were collected from a total of 30 marmosets from two research colonies of the common

marmoset (Southwest National Primate Research Center, Texas Biomedical Research Institute, San Antonio, Texas and the Barshop Institute for Longevity & Aging Studies University of Texas Health Science Center at San Antonio) and were used to establish reference intervals for concentrations of serum and fecal α_1-PI concentrations in healthy marmosets. Collection of samples was approved by the institutions' AUP protocols 1259CJ and 06120X, respectively. Paired fecal and serum samples were obtained to ensure that α_1-PI deficiency does not occur in individual common marmosets as has been described for humans.[78] Samples from 3 consecutive days were used because fecal α_1-PI concentrations in other species have been reported to show considerable day-to-day variations.[147] Fecal samples were collected into pre-weighed polypropylene tubes ([g]Fecal collection tube (101 X 16.5 mm; including spatula), Sarstedt AG & Co, Nümbrecht, Germany) as soon as possible after voiding and were stored at -80ºC until they were shipped on dry ice.

4.2.4 Extraction of fecal samples

Fecal samples were thawed to room temperature before extraction. They were extracted using phosphate buffered saline supplemented with 5% newborn calf serum, 1% Triton X-100, and 0.25 mM thimerosal in a 1:5 dilution, using approximately 1.0 g wet weight of feces. The samples were then vortexed for 20 minutes and then centrifuged for 20 min at 2,100 × *g* and 5°C. The supernatants (fecal extracts) were collected using serum filters (Fisherbrand serum filter system (IB model), Fisher Scientific Inc, Pittsburgh, PA), and were stored frozen at –80°C until further analysis.

4.2.5 Preparation of serum samples and fecal extracts

Serum samples were diluted 1:64,000 in 0.05 M sodium phosphate, with 0.02% sodium azide and 0.5% bovine serum albumin (pH 7.5). Previously stored fecal extracts were thawed and diluted in 0.05 M sodium phosphate, with 0.02% sodium azide and 0.5% bovine serum albumin (pH 7.5), in order to reach a final dilution of 1:200.

4.2.6 Preparation of standards

A 1 mg/ml solution of purified marmoset α_1-PI was diluted to concentrations of 100, 50, 20, 10, 5, 2, and 1 µg/L, using 0.05 M sodium phosphate, with 0.02% sodium azide and 0.5% bovine serum albumin (pH 7.5).

4.2.7 Enzyme-linked immunosorbent assay procedure

The following procedures were carried out at 37°C. Each well of a ninety-six-well, enhanced binding ELISA plate (MaxiSorp Nunc-Immuno Plates, Thermo Scientific) was incubated with 100 ng of affinity purified anti-marmoset α_1-PI antibodies diluted in 100 µL of 200 mM carbonate-bicarbonate, pH 9.4 and incubated for 1 hr at 37°C. Three washes with 200 µL of 25 mM Tris/HCl, 150 mM NaCl, 0.05% polyethylene glycol sorbitan monolaurate (TWEEN-20, Sigma–Aldrich, Inc., St. Louis, Missouri), pH 8.0 [wash buffer] were performed for each well. Next, 200 µL of 25 mM Tris/HCl, 150 mM NaCl, 0.05% polyethylene glycol sorbitan monolaurate, 10% bovine serum albumin, pH 8.0 was used for blocking the nonspecific binding sites [blocking buffer]. Plates were incubated again for 1 hr at 37°C, followed by 3 washes with wash buffer. Coated plates were stored at 4°C for up to 2 weeks.

All reagents and samples were thawed. A total of 100 μL of seven standard solutions, blank, and diluted test samples of serum or fecal extracts were applied to each plate in duplicate fashion and incubated for one hour at 37°C. For the blank, an equal volume of 25 mM Tris/HCl, 150 mM NaCl, 0.05% polyethylene glycol sorbitan monolaurate, 0.5% bovine serum albumin, pH 8.0 [assay buffer] was used. Three washes were performed with the wash buffer. The plates were then incubated with the HRP-conjugated antibody diluted in assay buffer (50 ng/well) for another hour at 37°C. The plates were then once again washed 3 times with the wash buffer. The plate was then developed with a 3,3′,5,5′-tetramethyl benzidine (1-Step Ultra TMB-ELISA, Thermo Scientific) substrate. After a 5-min incubation, the reaction was stopped by adding 4 M acetic acid 0.5 M sulfuric acid [stopping buffer]. The microtiter plate was then read at 450 nm using an automated plate reader (Synergy 2 Alpha Microplate Reader; BioTek, Winooski, VT, USA). A commercial software package (Gen5 Data Analysis Software v1.05; BioTek) was used to calculate a 5-parameter logistic curve fit (F(x) = D+(A-D)/((1+(x/C)^B)^E)], which was used to determine α_1-PI concentrations in the test samples.

During the initial developmental phase, several parameters of the assay were optimized for better performance including titration of the primary and secondary antibodies.

4.2.8 Assay validation

The ELISA was validated by determining the lower limit of detection (LLOD), dilutional parallelism, spiking recovery, intra-assay variability, and interassay variability. The validation procedures were performed separately for serum samples and fecal extracts. The lower detection limit of the assay was determined by loading 10 sets of blank controls as unknown samples and calculating the mean concentration plus 3 standard deviations for 20 replicates. Four serum

samples and four fecal extracts were used to determine dilutional parallelism. Two-fold dilution of serum samples from 1:64,000 to 512,000 and a two-fold dilution of fecal samples from 1,000 to 8,000 were used and the percentage of standard antigen recovery ([observed value (ng/g)/expected value (ng/g)] × 100) was calculated in each case. Spiking recovery was determined by adding 20, 50, and 100 µg/L of pure marmoset α_1-PI to four serum samples and four fecal extracts. The percentage of standard antigen recovery ([observed value (ng/g)/expected value (ng/g)] × 100) was calculated in each case. Intra-assay variability was determined by evaluating 4 serum samples and 4 fecal extracts multiple times within the same assay run. The intra-assay coefficient of variation (% CV) for each sample (%CV = [SD/mean] × 100) was calculated. Inter-assay variability was determined by evaluating 4 serum samples and 4 fecal extracts within multiple consecutive assay runs. The inter-assay coefficient of variation (%CV) for each sample (%CV = [SD/mean] × 100) was calculated.

4.2.9 Position effect

Four serum samples and 4 fecal extracts were randomized to 32 positions in duplicates on an ELISA plate using an online software (https://www.random.org/lists/). Concentrations were compared for the samples using coefficient of variation (%CV) for each sample (%CV = [SD/mean] × 100). A Kruskal–Wallis test by ranks was used to compare concentrations from these 4 positions for each sample. Commercially available software (GraphPad Prism v5.0, GraphPad Software, San Diego, CA, USA) was used for the statistical analysis and significance was set at $p<0.05$.

4.2.10 Reference interval

A reference interval for serum and fecal α_1-proteinase inhibitor concentrations (three-day mean and three-day maximum) was calculated using the central 95th percentile using serum samples and fecal extracts from healthy marmosets with no signs of chronic lymphocytic enteritis. Additionally, the mean coefficient of variation for the three fecal samples was calculated for this group of marmosets.

4.3 Results

Standard curves for the marmoset α_1-PI ELISA were reproducible (Figure 5). The lower detection limit of the assay was 0.006 µg/L.

Observed to expected ratios for serial dilutions for dilutional parallelism were for 4 serum samples were (minimum – maximum, mean ± SD): 112.2% – 123.0%, 117.1 ± 5.6% and were 82.6 – 130.2%, 106.1 ± 19.7% for 4 fecal extracts (Table 11, 12).

Observed to expected ratios for spiking recovery for 4 serum samples were (minimum – maximum, mean ± SD): 86.8 – 115.8%, 102.9 ± 12.1% and were 83.0 – 125.1%, 97.9 ± 19% for 4 fecal extracts (Table 13, 14).

Intra-assay variability coefficients of variation (CV %) for 4 serum samples were were 2.4, 2.4, 2.6, and 4.1% and were 2.6, 5.5, 6.6, and 6.8% for 4 fecal extracts (Table 15, 16).

Inter-assay variability coefficients of variation (CV %) for 4 serum samples were 1.7, 3.1, 3.1, and 6.3% and were 5.1, 8.1, 10.4, and 13.8% for 4 fecal extracts (Table 17,18).

No significant position effect was appreciated on the ELISA plate (p=0.9635). The median %CV between the positions was 3.5% (minimum to maximum; 2.3% to 6.8%).

A reference interval for serum and fecal α_1-proteinase inhibitor concentrations (three-day-mean and three-day-maximum) was calculated using the central 95th percentile for serum samples and fecal extracts from healthy control marmosets The reference interval for serum concentrations was 1,254 -1,813 mg/L, 11.5-42.2 µg/g for the three day mean fecal concentration, and 13.2 - 51.2 µg/g for the three day maximum fecal concentration (Table 19).

The median coefficient of variation of marmoset α_1-PI concentrations in fecal extracts between the three fecal samples was 23.0% (range: 3.3 – 77.0%).

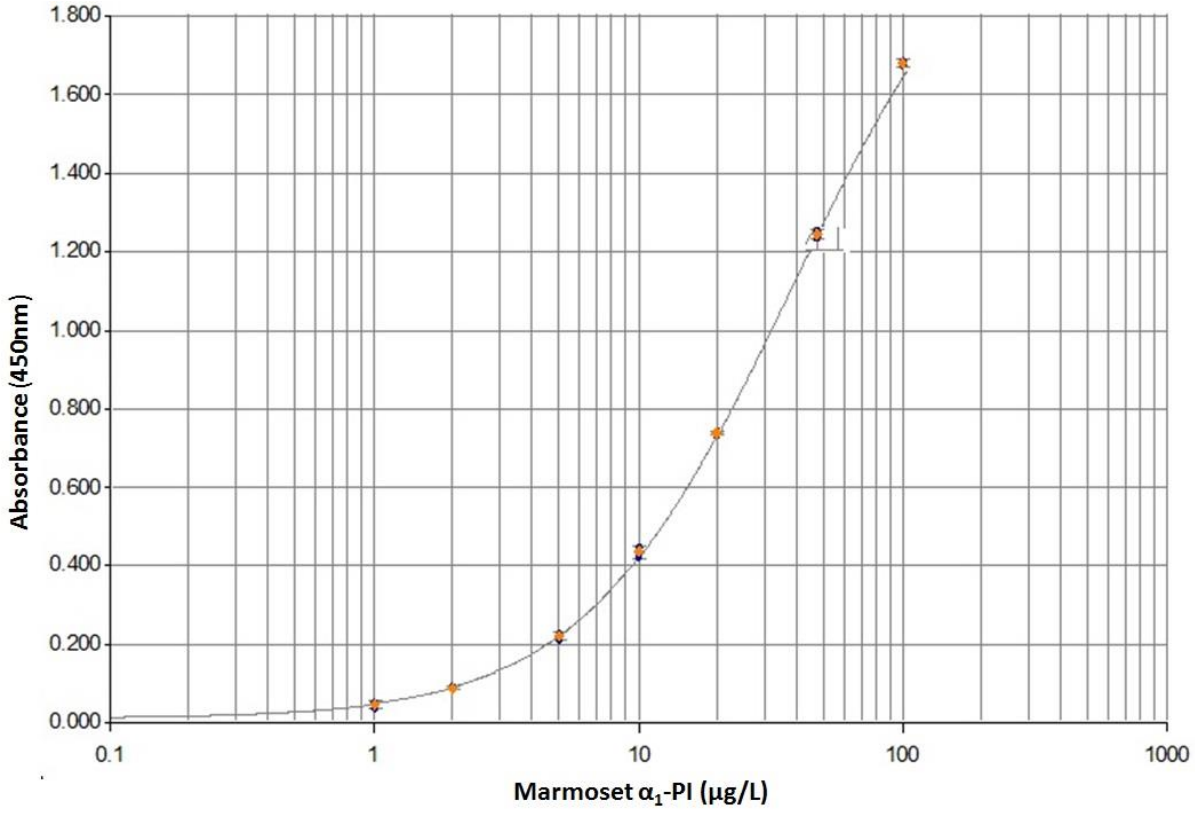

Figure 5. Representative standard curve for the marmoset α_1–PI ELISA. The curve is based on a five-parameter logistic fit. The seven standards had the following concentrations: 100, 50, 20, 10, 5, 2, and 1 µg/L. Reprinted with permission.[117]

Table 11. Results of dilutional parallelism for the marmoset serum α_1–PI ELISA shown for 4 serum samples at dilutions of 1 in 64,000 to 1 in 512,000. Reprinted with permission.[117]

Sample ID	Dilution	Observed Concentration (µg/g)	Expected Concentration (µg/g)	O/E%	Mean
Serum 1	64,000	18.3	N/A	N/A	
	128,000	10.0	9.2	109.5	
	256,000	5.2	4.6	114.4	
	512,000	2.6	2.3	113.6	112.5
Serum 2	64,000	18.7	N/A	N/A	
	128,000	10.5	9.4	112.5	
	256,000	5.4	4.7	115.5	
	512,000	2.5	2.3	108.5	112.2
Serum 3	64,000	19.5	N/A	N/A	
	128,000	11.0	9.7	112.6	
	256,000	6.2	4.9	126.7	
	512,000	3.2	2.4	129.8	123.0
Serum 4	64,000	17.4	N/A	N/A	
	128,000	10.0	8.7	115.3	
	256,000	5.3	4.4	121.7	
	512,000	2.7	2.2	124.7	120.6

O/E — observed/expected

Table 12. Results of dilutional parallelism for the marmoset fecal α_1-PI ELISA shown for 4 fecal samples at dilutions of 1 in 1,000, to 1in 8,000. Reprinted with permission.[117]

Sample ID	Dilution Factor	Observed Concentration (µg/g)	Expected Concentration (µg/g)	O/E%	Mean
Fecal 1	1,000	7.8			
	2,000	3.9	3.9	100.1	
	4,000	1.8	1.9	93.4	
	8,000	0.5	1.0	54.2	82.6
Fecal 2	1,000	24.9			
	2,000	15.4	12.5	123.6	
	4,000	8.4	6.2	134.5	
	8,000	4.1	3.1	132.6	130.2
Fecal 3	1,000	17.4			
	2,000	9.9	8.7	114.5	
	4,000	5.0	4.3	114.6	
	8,000	2.2	2.2	100.8	110.0
Fecal 4	1,000	12.2			
	2,000	6.9	6.1	112.9	
	4,000	3.4	3.0	112.6	
	8,000	1.2	1.5	79.0	101.5

O/E — observed/expected

Table 13. Results of the spiking recovery for the for marmoset serum α_1-PI ELSIA shown for 4 serum samples spiked with 3 concentrations. Reprinted with permission.[117]

Sample ID	Concentration added (µg)	Observed Concentration (µg/g)	Expected Concentration (µg/g)	O/E %	Mean
Serum 1	0	17.6			
	10	29.1	27.6	105.4	
	25	48.4	42.6	113.7	
	50	86.8	67.6	128.4	115.8
Serum 2	0	21.2			
	10	32.4	31.2	104.2	
	25	49.2	46.2	106.7	
	50	76.6	71.2	107.7	106.2
Serum 3	0	34.0			
	10	45.0	44.0	102.3	
	25	60.0	59.0	101.8	
	50	87.2	84.0	103.8	102.6
Serum 4	0	32.7			
	10	38.5	42.7	90.2	
	25	50.5	57.7	87.4	
	50	68.5	82.7	82.8	86.8

O/E — observed/expected

Table 14. Results for spiking recovery for the for marmoset fecal α_1-PI ELSIA shown for 4 fecal extracts spiked with 3 concentrations. Reprinted with permission.[117]

Sample ID	Concentration added (µg)	Observed Concentration (µg/ g)	Expected Concentration (µg/ g)	O/E %	Mean
Fecal 1	0	10.0			
	10	23.4	20.0	117.0	
	25	43.2	35.0	123.4	
	50	81.0	60.0	135.0	125.1
Fecal 2	0	60.2			
	10	73.1	70.2	104.2	
	25	82.9	85.2	97.4	
	50	96.9	110.2	88.0	96.5
Fecal 3	0	10.9			
	10	18.0	20.9	85.8	
	25	28.7	35.9	80.0	
	50	50.7	60.9	83.2	83.0
Fecal 4	0	43.1			
	10	52.4	53.1	98.8	
	25	59.7	68.1	87.7	
	50	69.7	93.1	74.9	87.1

O/E — observed/expected

Table 15. Precision of the ELISA for serum marmoset α_1–PI (intra-assay validation). Reprinted with permission.[117]

	Concentration of Marmoset α_1 - Proteinase inhibitor (µg/g)									
Sample ID	1	2	3	4	5	6	7	8	Mean	CV%
Serum 1	32.3	33.8	32.8	33.6	33.2	33.0	32.6	31.1	32.8	2.6
Serum 2	35.8	37.7	37.4	36.7	35.3	35.9	36.2	37.2	36.5	2.4
Serum 3	37.6	38.8	37.6	36.4	36.3	37.8	37.1	36.2	37.2	2.4
Serum 4	33.6	35.0	35.1	37.1	33.7	33.4	34.5	36.8	34.9	4.1

CV %: coefficient of variation

Table 16. Precision of the ELISA for fecal marmoset α_1–PI (intra-assay validation). Reprinted with permission.[117]

	Concentration of Marmoset α_1 - Proteinase inhibitor (µg/g)									
Sample ID	1	2	3	4	5	6	7	8	Mean	CV%
Fecal 1	21.0	21.3	21.6	21.3	20.7	21.1	20.2	20.1	20.9	2.6
Fecal 2	58.2	57.6	59.0	63.4	55.9	56.9	61.0	67.9	60.0	6.6
Fecal 3	30.3	30.5	29.4	28.4	28.3	29.1	26.9	26.0	28.6	5.5
Fecal 4	20.8	21.4	21.7	23.6	19.6	20.9	22.2	24.0	21.8	6.8

CV : coefficient of variation

Table 17. Reproducibility of the ELISA for serum marmoset α_1–PI (inter-assay validation). Reprinted with permission.[117]

	Concentration of Marmoset α_1 - Proteinase inhibitor (µg/g)					
Sample ID	Run 1	Run 2	Run 3	Run 4	Mean	CV (%)
Serum 1	24.7	25.4	26.6	26.0	25.7	3.1
Serum 2	28.2	32.7	29.9	29.3	30.0	6.3
Serum 3	29.7	30.7	30.9	30.7	30.5	1.7
Serum 4	30.1	30.3	30.4	28.4	29.8	3.1

CV %: coefficient of variation

Table 18. Reproducibility of the ELISA for fecal marmoset α_1–PI (inter-assay validation). Reprinted with permission.[117]

	Concentration of Marmoset α_1 - Proteinase inhibitor (μg/g)					
Sample	Run 1	Run 2	Run 3	Run 4	Mean	CV (%)
Fecal 1	20.0	20.6	23.6	20.2	21.1	8.1
Fecal 2	52.4	50.0	67.6	56.8	56.7	13.8
Fecal 3	29.3	30.1	36.2	29.7	31.3	10.4
Fecal 4	20.3	20.4	22.2	22.3	21.3	5.1

CV %: coefficient of variation

Table 19. Reference intervals established from 30 healthy marmosets using the developed ELISA for concentrations of marmoset α_1–PI in serum and feces. Reprinted with permission.[117]

	Concentration of marmoset α_1 - PI		
	Serum concentration (mg/L)	Three day mean fecal concentration (µg/g)	Three day maximum fecal concentration (µg/g)
Reference interval	1254.3-1813.5	11.5-42.2	13.2 - 51.2
Median ± SD	1525.1 ± 158.1	23.8 ± 9.0	29.6 ± 11.5
Range	1203.0 – 1975.1	9.9 - 46.7	11.4-53.4

4.4 Discussion

An ELISA for the quantification of marmoset α_1-PI in serum samples and fecal extracts was successfully developed. The assay was determined to be precise and reproducible for both types of biological samples using currently accepted criteria.[160] However, the assay does have its limitations with respect to linearity and accuracy. Mild deviations (an O/E ratio >120%, but less than 130%) from the generally acceptable range of 80% - 120% were observed for dilutional parallelism and spiking recovery of serum samples. However, more pronounced deviations were seen for fecal samples, for both dilutional parallelism and spiking recovery. This discrepancy in performance between serum samples and fecal extracts may be attributable to a possible matrix effect when using fecal extracts. The greatest underestimation of α_1-PI concentrations occurred when the expected concentration was less than 2 ng/g of feces during dilutional parallelism (O/E% of 54.2%, and 79%); given that the intent of the assay was to detect increases in fecal concentrations of marmoset α_1-PI as evidence of gastrointestinal protein loss, issues with linearity in samples with a concentration less than 2 ng/g of feces should not detract from the clinical usefulness of the assay. The specific reason for over-recovery of this assay is unknown. However this problem is generally attributed to the presence of high-affinity antibodies.[14]

The reference interval for concentrations of marmoset α_1-PI in serum was from 1,254 - 1,814 mg/L. This is comparable with serum α_1-PI concentration reported in other species such as the dog, cat, and human, which are 732-1802 mg/L,[59] 250-600mg/L,[44] and 900-1200mg/L,[93] respectively.

Fecal α_1-PI concentrations in healthy cats are reported to be from 0.04 (or undetectable) to 1.72 µg/g (median: 0.51 µg/g) and in healthy adult dogs from <2.2 to 26.8 µg/g (median: 4.7 µg/g). In the 30 healthy marmosets in this study fecal α_1-PI concentrations were much higher and ranged

from 11.4 to 48 µg/g (median: 27.1 µg/g) for samples collected on day 1. A reason for this increased concentration is unknown.

As reported for dogs[58] and cats[26], a similarly high %CV was also observed in the common marmoset within the 3-day fecal samples (median: 26.3%, minimum-maximum 3.4% - 77.0%), i.e., there is considerable day to day variation of fecal α_1-PI concentrations. This justifies the need for a 3 consecutive fecal collections in this species.

An important limitation of this study is the low number marmosets in the reference sample group for both fecal extracts and serum samples. Ideally, reference intervals are calculated from results from approximately 120 healthy individuals. However, samples form 30 marmosets were all that was available to us. As samples were limited, we did not further investigate the effect of storage or repeated freeze-thaw cycles on the recovery of marmoset α_1-PI concentrations in serum samples or fecal extracts. As there were only limited numbera of samples available for validation purposes a relatively narrow range of concentrations was represented that did not span the whole working range of the assay. Validation with samples with a wider range would have been preferable, but this was not feasible.

In conclusion, the marmoset α_1-PI ELISA described here is sensitive, precise, and reproducible with suboptimal linearity for fecal samples less than 2 ng/g, and suboptimal accuracy, as assessed by spiking recovery, at the either end of the assay working range. Additional clinical studies are necessary to determine the clinical utility of this assay for detecting protein losing enteropathy in marmosets.

CHAPTER V

FECAL ALPHA$_1$-PROTEINASE INHIBITOR CONCENTRATIONS IN COMMON MARMOSETS (*CALLITHRIX JACCHUS*) WITH CHRONIC LYMPHOCYTIC ENTERITIS.

5.1 Introduction

The common marmoset (*Callithrix jacchus*) is a New World primate originally from Brazil. Its small size, easy maintenance, absence of certain zoonotic diseases, multiple births at each parturition, and relatively short lifespan make it a popular species for biomedical research.[90,1] Chronic lymphocytic enteritis (CLE) is endemic to marmoset research colonies, with the reported prevalence varying from 28–60 % in the laboratory setting.[88] Clinically, CLE it is associated with chronic progressive weight loss in adults and unthriftiness in juveniles. Diarrhea, mild anemia, and low serum protein concentrations are common, with affected marmosets either dying or being euthanized.[11,88] The disease currently can only be confirmed upon necropsy.

A non-invasive fecal test to detect enteric protein loss may allow early ante-mortem diagnosis and could facilitate screening and removal of affected marmosets in research colonies. Alpha$_1$-proteinase inhibitor (α_1-PI) is a serum protein that is only lost into the gastrointestinal tract in minimal quantities in healthy individuals. It's molecular weight is similar to that of albumin, but it is resistant to degradation by digestive and bacterial enzymes within the gut. Intact α_1-PI can be detected by fecal immunoassays and the assay has previously been employed to detect increased enteric protein loss in humans, [22] dogs,[96] and cats.[25] Recently, two immunoassays, a radioimmunoassay (RIA) and a sandwich enzyme-linked immunosorbent assay (ELISA) for measuring the concentrations of α_1-PI in serum and fecal extracts from the common marmoset have been developed and analytically validated.

The aim of this study was to determine if fecal concentrations of α_1-PI were increased in marmosets with CLE, suggesting a protein losing enteropathy.

5.2 Materials and methods

5.2.1 Agreement between the RIA and the ELISA

To assess the agreement between results obtained by the newly developed immunoassays, RIA and ELISA, the results of fecal α_1-PI concentrations from 47 common marmosets from three different primate centers [NEPRC (n=17), Southwest National Primate Research Center; Texas Biomedical Research Institute, Texas (n=17), and the Barshop Institute for Longevity & Aging Studies University of Texas Health Science Center, Texas (n=14)] were compared. The concentrations of fecal α_1-PI obtained in the RIA were plotted against those determined using the ELISA, and a Spearman correlation coefficient (ρ) was calculated. This was followed by a Tukey mean-difference plot (Bland–Altman plot) to look for biases. Based on these results, one immunoassay was chosen for the study.

5.2.2 Marmoset fecal samples

Fecal samples from marmosets previously submitted to the Gastrointestinal Laboratory at Texas A&M University for research purposes (n=10) and fecal samples collected at necropsy (n=15) from common marmosets euthanized as a part of routine colony management procedures at the New England Primate Research Center (NEPRC), Southborough, MA were stored at -80^{o}C and shipped on dry ice and were used for the study.

Clinical history and necropsy records were obtained and reviewed for these animals to ascertain cause of death. This was done after the assay was completed, in order to avoid any bias. Necropsies were performed by a board certified anatomic pathologist at the NEPRC.

5.2.3 Extraction of fecal samples and RIA

Fecal samples were thawed and then extracted as previously described to generate the fecal extract (Chapter 3). Fecal extracts were stored at –80°C until analysis. Stored fecal extracts were thawed and diluted 1:200 in radioimmunoassay buffer. Samples were run using the previously validated marmoset α_1-PI radioimmunoassay procedure (Chapter 3). The results were compared to the previously established, three-day mean fecal α_1-PI concentration reference interval.

5.2.4 Statistical analyses

Statistical analyses were performed using GraphPad Prism 5.0 [GraphPad Software, Inc., San Diego, CA) and statistical significance was set at $P < 0.05$.

5.3 Results

The concentrations of fecal α_1-PI measured in 47 marmoset samples by use of the RIA were only moderately positively correlated to the results of the ELISA as indicated by a Spearman ρ of 0.71 ($P < 0.0001$) [Figure 6]. The difference between both immunoassays was plotted against their mean (Bland–Altman plot; Figure. 7) with a mean difference of -20. Given the discrepancy between the two immunoassays for the same samples and the validation of the RIA being more robust compared to the ELISA, we decided to use the RIA to determine if marmosets with CLE had increased concentrations of fecal α_1-PI.

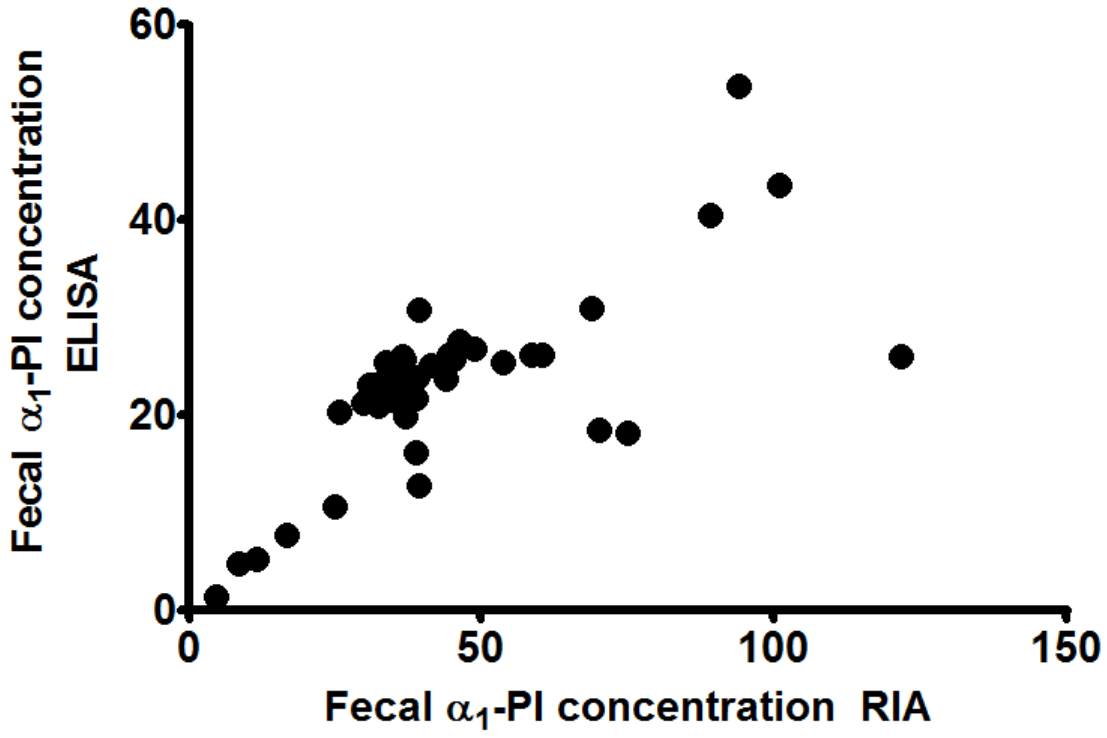

Figure 6. Correlation of fecal α_1-PI concentrations (µg/g) measured in 47 fecal samples from marmosets using both the radioimmunoassay and the ELISA. The two assays were moderately correlated (Spearman ρ of 0.71, P <0.0001).

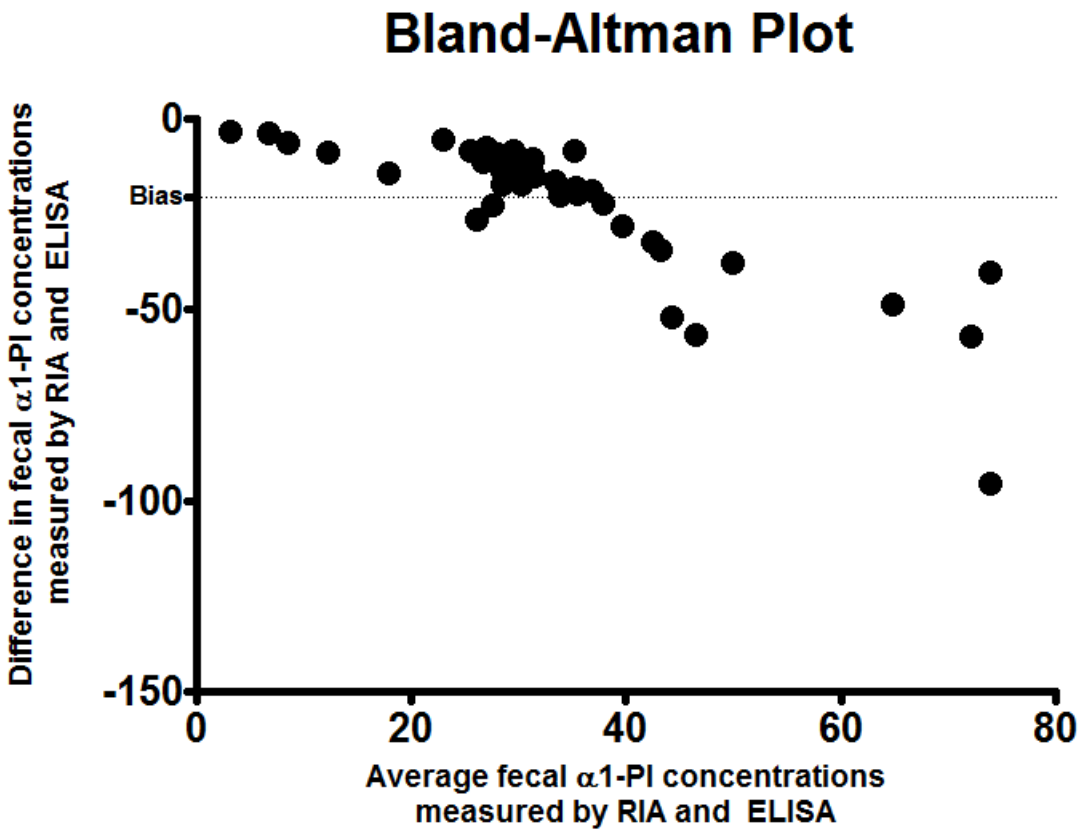

Figure 7. Bland–Altman plot. Each symbol represents the difference between both methods against their mean for a specific fecal sample (n = 47). The mean difference (bias) was calculated as -20.46 (95% confidence interval [CI]: -54.99 to 14.08).

For a total of 25 marmosets fecal α_1-PI concentrations and necropsy findings were available, and among those 15 also had a clinical history available. Of these marmosets, 11 had CLE, 9 had other diseases (i.e., chronic kidney disease (5), cholecystitis (2), pancreatitis (1), lymphoma and ulcerative enteritis (1), enteropathogenic E. Coli (1), and small intestinal adenocarcinoma (1)), and 5 were healthy. Fecal α_1-PI concentrations were not significantly different between the three groups (Kruskal–Wallis test, $P = 0.50$).

Other diseases observed in these marmosets included conditions, such as intestinal adenocarcinoma, that could also lead to an intestinal protein loss, thereby increasing fecal α_1-PI concentrations. The marmosets were again regrouped into healthy marmosets (5), those with diseases that might be associated with intestinal protein loss (14), and those with diseases not associated with intestinal protein loss (6). Fecal α_1-PI concentrations were not significantly different between these three groups (Kruskal–Wallis test, $P = 0.75$).

Only one marmoset with renal disease had a fecal α_1-PI concentration greater than the 3-day-mean of 234.0 µg/g of feces as reported in healthy marmosets. Necropsy findings on this marmoset's intestinal tract showed one section of the proximal jejunum with a single dilated crypt filled with cellular debris and few degenerate neutrophils. The cecum and the colon showed minimal to low numbers of lymphocytes and plasma cells within the mucosa, and the cecum had multifocal areas of mild crypt regeneration.

5.4 Discussion

This study failed to show a significant increase in fecal concentrations of α_1-PI in common marmosets with chronic lymphocytic enteritis, based on previously established reference intervals for the common marmoset. These findings are in contrast to dogs where increased fecal

concentrations of α_1-PI have been reported in patients with gastrointestinal disease, particularly those with IBD and lymphangectasia,[107] acute hemorrhagic diarrhea syndrome,[57] parvoviral gastroenteritis,[104] tylosin responsive diarrhea,[164] and also in healthy sled dogs after a race.[39] In cats, increased fecal concentrations of α_1-PI have been reported in patients with severe IBD or confirmed gastrointestinal neoplasia.[25] In humans, increased fecal concentrations of α_1-PI have been reported in a variety of gastrointestinal disorders,[149] and also as an indicator of Crohn's disease activity.[99] A good correlation has been reported between the gold standard, ^{51}Cr-albumin excretion and fecal α_1-PI in humans[123] and in dogs.[94] However, it has been reported to have a poor sensitivity (58%), with a good specificity (80%), limiting its use in humans.[123] It remains unknown if lower concentrations of fecal α_1-PI in the common marmoset are a result of this low sensitivity.

Normal concentrations of fecal α_1-PI were unexpected in this study. It has previously been shown that increased gastric acid secretion can lead to decreased fecal α_1-PI concentrations. However, this would seem to be an unlikely explanation in the marmosets as marked increased gastric acid secretions would have led to lesions in the stomach, that would have been observed upon necropsy.

Alpha$_1$-PI in humans is excreted in the feces in two forms, a protease-antiprotease complex, but also as an unchanged α_1-PI when compared to serum.[24] The proportion of these two products are variable between individuals and an increased presence of protease-antiprotease complex might lead to a false decrease in the apparent fecal α_1-PI concentration. This effect has been observed in an in-vitro study using canine α_1-PI spiked with bovine trypsin.[95]

It is interesting to note that in the one marmoset with renal failure, elevated fecal α_1-PI concentrations with no, or minimal changes of gastrointestinal histopathologic morphology did have decreased serum cobalamin and folate concentrations of <150 pg/mL and 28.3 ng/mL,

respectively. These results are much lower than the established reference intervals of 322 – 2,642 pg/mL and 54.8 – 786.4 ng/mL for serum cobalamin and folate concentrations, respectively. Decreased serum cobalamin and folate concentrations do suggest diffuse small intestinal disease, but histological confirmation was not achieved in this case. A focal area with ulceration or inflammation leading to enteric protein loss could still be present, and it could have been in an area of gut that was not sectioned. The clinical history of this marmoset also indicated progressive weight loss and a chronic anemia for over a year, both of which have been reported in marmosets with CLE. However, these clinical signs are non-specific and can also be associated with chronic kidney disease in older marmosets. The marmoset was 4,667 days, approximately 13 years, old, which is the approximate life span of common marmosets in captivity.

The RIA validation study (Chapter 3) did not explore the effect of storage conditions on the concentrations of fecal α_1-PI in the marmoset, and assumed that freezing helped to preserve fecal α_1-PI in the common marmoset, which was extrapolated from canine studies.[96] Another factor for the lack of increased fecal α_1-PI concentrations detected in this study could be that while the feces remained in the animal after euthanasia or death, the protein in the fecal sample could have been denatured or destroyed by the body's autolytic process. The exact time from death to necropsy was unknown, with the median being zero days but it ranged from 0-3 days based on medical records. All necropsies had an associated form with a check box to indicate degree of autolysis, and all were marked as fresh. Interestingly, the marmoset with greatest concentration of fecal α_1-PI, had the greatest time recorded of three days, suggesting that a falsely decreased fecal α_1-PI concentration because of fecal collection at delayed necropsy was an unlikely cause for lower α_1-PI concentrations.

Fecal samples used in this study were collected from the rectum at necropsy and frozen, versus naturally passed samples used to establish the reference interval in healthy marmosets. It has been shown in dogs that rectal collection[159] does increase fecal α_1-PI concentrations, presumably due to the presence of blood. However, this is controversial in the human literature.[54,156] Given the lack of increase in fecal α_1-PI concentrations in these marmosets, it is unlikely that the method of collection affected these results.

Only a limited number of samples were available to establish the earlier reference intervals for fecal α_1-PI (Chapter 3) and they were primarily from two different centers other than the NEPRC. Animals that were used to establish the reference interval were presumed to be healthy based on the absence of clinical signs. However, given how little is known about the disease, subclinical chronic lymphocytic enteritis could not be ruled out as gastrointestinal biopsies were not available. Thus, it is possible that the reference interval may have to be revisited.

Limitations of this study include a single time point for fecal sample collection, which may not have been appropriate given the relatively high coefficient of variation (median 21.4%, Range 2.9 – 74.3%) between the three-day fecal samples reported in the earlier study, and this has also been reported in other species such as the dog.[58] Larger studies looking at single time point and three-day fecal collections should be performed. Serum protein, and albumin concentration data were also not available for these marmosets.

To conclude, fecal α_1-PI concentrations were not significantly increased in a small population of common marmosets with CLE. Larger studies are needed to determine the utility of fecal α_1-PI concentrations in marmosets with gastrointestinal disease, particularly CLE.

CHAPTER VI

SERUM COBALAMIN AND FOLATE CONCENTRATIONS IN COMMON MARMOSETS (*CALLITHRIX JACCHUS*) WITH CHRONIC LYMPHOCYTIC ENTERITIS.[∞]

6.1 Introduction

The common marmoset (*Callithrix jacchus*) is a Brazilian New World monkey that has been employed in biomedical research since the early 1970's.[1] Marmosets are a popular species for biomedical research because of their small size (350-450g), relatively low cost for maintenance, easy husbandry, easy habituation to routine clinical procedures, rapid reproductive turnover, and low incidence of zoonoses. Currently, marmosets are used for research in the fields of neuroscience, reproductive biology, infectious diseases, behavior, drug development and safety assessment, and aging research.[90]

Inflammatory diseases of the intestinal tract, particularly colitis and inflammatory bowel disease (IBD), particularly chronic lymphocytic enteritis (CLE) has been a consistent finding in several colonies of marmosets.[32,38,158] Between 1991 and 2000, about 60.5% of all non-experimental marmosets at the New England Primate Research Center (NEPRC), Southborough, MA had some degree of IBD.[154] Similarly, from 2002-2011 at the Southwest National Primate Research Center, Texas Biomedical Research Institute, San Antonio (SNPRC) facility, 31%-44% of marmoset deaths were attributed to IBD.[131] To date, no specific etiology has been proven for this disease in the marmoset. However, many factors such as gluten sensitivity, dietary protein deficiency, and pancreatic duct parasitism (*Trichospirura leptostoma*) have been speculated to

[∞]Submitted to Comparative Medicine. Parambeth JC, Ross C, Miller A, Austad SN, Lidbury JA, Suchodolski JS, Steiner JM. Serum cobalamin and folate concentrations in common marmosets (*Callithrix jacchus*) with chronic lymphocytic enteritis.

play a causal role.[27] CLE is characterized histologically by diffuse to segmental lymphocytic enteritis. Clinically, it manifests itself by failure to thrive in juveniles or weight loss in adults with or without diarrhea.[88] Currently, a presumptive ante-mortem diagnosis is made based on consistent clinical signs, a history of weight loss, and a decreased serum albumin concentration.[11] Fecal calprotectin,[108] serum matrix metalloproteinases,[171] serum IgA antibodies against gliadin and related proteins[84] have been measured in marmosets with CLE and may serve as diagnostic markers in the future. To date, no treatment has been shown to be effective and a definitive diagnosis is usually made at necropsy.[88]

Cobalamin (vitamin B12) is a water soluble vitamin that is an essential co-factor for the enzymes methylmalonyl CoA mutase, methionine synthase, and methylmalonyl-coA mutase. Intestinal uptake of cobalamin is a multistep process, with absorption of cobalamin-intrinsic factor complexes occurring through specific receptors on ileal enterocytes.[91,150] Cobalamin deficiency is reported frequently in dogs and cats with exocrine pancreatic insufficiency, distal small intestinal disease, or diffuse small intestinal disease, and is less commonly attributed to genetic defects in certain dog breeds.[47] Exocrine pancreatic insufficiency causes hypocobalaminemia in dogs as the majority of intrinsic factor in dogs is synthesized by pancreatic acinar cells.[9,35] Ileal disease is believed to lead to damage or decreased expression of cobalamin receptors, ultimately leading to reduced cobalamin absorption and deficiency once cobalamin body stores have been used up. Intestinal dysbiosis can also lead to decreased serum cobalamin concentrations as many bacterial species compete for and bind available cobalamin.[49] The reported prevalence of cobalamin deficiency in dogs and cats with gastrointestinal disease ranges from 6 - 18.5% in dogs[5,35] and from 17 - 61% in cats.[128,141] Furthermore, hypocobalaminemia has been associated with negative outcomes in dogs with chronic enteropathy[5] or exocrine pancreatic insufficiency,[8] and cobalamin

supplementation may have therapeutic potential.[132] Decreased serum cobalamin concentrations have been reported in cats with exocrine pancreatic insufficiency,[168] IBD, and gastrointestinal neoplasia such as lymphoma and adenocarcinoma.[71] To the best of our knowledge, no data on the cobalamin status of marmosets has been reported to date. However, recently serum cobalamin concentrations have been investigated in rhesus macaques and pigtailed macaques with chronic idiopathic diarrhea.[21] Serum cobalamin concentrations were 2.5 fold lower in pigtailed macaques with chronic idiopathic diarrhea compared to healthy controls. However, no difference was observed in rhesus macaques.[21]

Folate (B9) is also a water-soluble B complex vitamin. Dietary folate in the form of folate polyglutamate is poorly absorbed in the intestinal tract. However, the enzyme, folate deconjugase in the jejunal brush border converts folate polyglutamate into monoglutamate, which is then absorbed into enterocytes of the proximal small intestine utilizing specific carriers. Chronic intestinal disease damages folate carriers, reducing folate uptake, and thus leading to decreased serum concentrations of folate.[146] Increased serum folate concentrations are reported in dogs with intestinal dysbiosis as many bacterial species synthesize folate.[49] In addition, chronic enteropathy also can lead to decreased serum folate concentrations in dogs and cats.[35,128] To the best our knowledge, serum folate concentrations have not been evaluated in marmosets with or without gastrointestinal disease.

Dietary deficiency of cobalamin and folate is highly improbable, and it is believed that even withholding food for several weeks duration does not cause serum cobalamin and folate to become subnormal.[146] Rather, characterizing the changes in serum concentrations of cobalamin and folate in marmosets with CLE may help facilitate monitoring gastrointestinal health in this species. The objective of this study was 1) to partially validate commercially available

immunoassays for the measurement of cobalamin and folate in human serum for use in the common marmoset, 2) to establish reference intervals for serum concentrations of cobalamin and folate in healthy common marmosets, and 3) to study the changes in the concentration of serum cobalamin and folate in marmosets with CLE.

6.2 Materials and Methods

6.2.1 Marmoset serum samples and data

This study was approved by the respective primate centers' Institutional Animal Care Committees. Serum samples were collected from common marmosets from the Southwest National Primate Research Center (SNPRC), Texas Biomedical Research Institute, San Antonio, Texas (AUP # 1259CJ), and the Barshop Marmoset Aging Center (BMAC), The University of Texas Health Science Center, San Antonio, Texas (06120X). Additionally, left over serum samples submitted to the Gastrointestinal Laboratory, Texas A&M University as part of diagnostic testing from the common marmoset colony at the New England Primate Research Center (NEPRC), Southborough, MA were also used in the study. Serum samples were transported on dry ice to the Gastrointestinal Laboratory, and stored at -80^{o}C until analysis.

Clinical history, and/or necropsy data from these common marmosets were reviewed to determine if they were healthy at the time of collection, had clinical signs of gastrointestinal disease, or had histopathological evidence of CLE.

6.2.2 Analysis of serum cobalamin and folate concentrations

Serum cobalamin and folate concentrations were measured using competitive chemiluminescence enzyme immunoassays (IMMULITE® 2000, Siemens, IL) approved for use

in humans. The reportable range of the cobalamin assay is 150-1,000 pg/mL and the reportable range for the folate assay is 1-24 ng/mL. Samples that had results over this reportable range were diluted with the sample diluent (# L2FVZ) supplied with the assay kits as per the manufacturer's recommendations.

6.2.3 Partial validation

Since no validation data was available for non-human primates, a partial validation for the assays was performed. Due to the small serum sample volumes available from individual common marmosets, serum samples were pooled to obtain sufficient volumes for assay validation. Four pooled serum samples were used for analytical validation.

Linearity of the assay was determined by dilutional parallelism. Four pooled serum samples were serially diluted (1:2, 1:4, 1:8, and 1:16) manually. The observed to expected ratio was calculated and expressed as a percentage (% O/E). The Immulite instrument is able to perform sample dilutions during runs, and this was included as a part of validation too. Four individual serum samples were run using dilutions available on the Immulite of 1:3, 1:5, 1:10, and 1:20 for the cobalamin validation. Five individual serum samples were run using dilutions available on the Immulite of 1:10, 1:20, 1:40, and 1:100 for the folate validation. The % coefficient of variation (CV% = standard deviation / mean*100) was reported by the Immulite.

Accuracy of the assay was determined by mixing three pooled samples with other pooled samples of known concentrations in a 1:1 ratio. Standard recovery was calculated from the ratio of observed concentration to expected concentration expressed as a percentage (% O/E). Spiking was also carried out using three QC controls provided with the assay in a 1:1 pattern to four serum

samples for the cobalamin assay and five serum samples for the folate assay. The observed-to-expected ratios were calculated as before.

Precision was determined by evaluating 4 pooled serum samples, 5 times on the same run, on the same day. Reproducibility was determined by evaluating 4 pooled serum samples, 5 times on different runs on consecutive days. Both precision and reproducibility were assessed by calculating the coefficient of variation (CV% = standard deviation / mean*100).

6.2.4 Stability testing

To assess the stability of serum cobalamin and folate samples stored at -80^{o}C, six samples analyzed in 2006 for serum cobalamin and folate concentrations were rerun in 2013.

6.2.5 Reference intervals

Reference intervals for serum cobalamin and folate were established by utilizing RefVal 2.1 (Department of Clinical Chemistry, Rikshospitalet, N-0027 Oslo, Norway) adapted for Microsoft Excel (Microsoft, Redmond, WA, USA), using samples from healthy marmosets with no previous history of gastrointestinal disease based on clinical history from two centers (BMAC & SNPRC).

6.2.6 Additional analysis

Metadata from the two primate centers (BMAC & SNPRC) was analyzed for serum concentrations of cobalamin and folate; particularly looking at location of housing, age in days (when sample was collected), sex, dam, sire, birthplace, litter size, weaning litter size, adult body weight, obesity status (as determined as function of body weight), and age group. Marmosets were

divided based on their age groups into infant (0 – 5 months), juvenile (5 – 10 months), sub-adult (10 – 15 months), and young adult (> 15 months).[170]

Serum cobalamin and folate concentrations were compared between healthy marmosets and those with any clinical history of gastrointestinal disease based on medical records from these two centers. Repeated measurements of serum cobalamin and folate from the same animals where available were analyzed over fixed time points.

6.2.7 Analysis using samples from the NEPRC

The established reference intervals were used to assess concentrations of serum cobalamin and folate in another marmoset colony (NEPRC), where cross sectional and longitudinal measurements were already available. Based on necropsy diagnosis, marmosets from the NEPRC were categorized as healthy, having CLE, or other diseases as the cause of death. Serum cobalamin and folate concentrations were compared between these three groups.

6.2.8 Sensitivity and specificity

Sensitivity and specificity were calculated using an online calculator (https://www.medcalc.org/calc/diagnostic_test.php). Sensitivity was calculated by the formula: a / (a+b) X 100, and specificity was calculated by the formula: d / (c+d) X 100; where a = marmosets with CLE and a low serum cobalamin and/or folate concentration, b = marmosets with CLE with normal serum cobalamin and folate concentrations, c = marmosets without CLE, but with a low serum cobalamin and/or folate concentrations, and d = marmoset without CLE and normal serum cobalamin and folate concentrations.

6.2.9 Statistics analysis

Statistical analyses were performed using commercially available software packages (JMP 10, SAS Institute Inc., Cary, NC and GraphPad PRISM 5.00, GraphPad Software, Inc. La Jolla, CA). Significance was set at $P<0.05$. Normality was tested using the Shapiro–Wilk test, which was not met. Where multiple parameters were evaluated, a Bonferroni correction was applied. Wilcoxon signed-rank test was used for repeated measures. Mann-Whitney U and Kruskal-Wallis tests with a Dunn's post-test were used to compare between groups.

6.3 Results

6.3.1 Validation

For the cobalamin measurements: observed-to-expected ratios for 1:1, 1:2, and 1:4 dilutions ranged from 113.2 to 282.2% (mean ±SD: 176.21 ±67.29 %) (Table 20). The CV% for the concentrations of serum cobalamin using dilutions available on the Immulite were 3.8%, 5.8%, 7.0%, and 8.8% for the 4 serum samples at three different dilutions at 1:3, 1:5, and 1:10. At a dilution of 1:20, the samples were too dilute and hence failed to give a result (Table 21). Observed-to-expected ratios for spiking recovery ranged from 91.5 to 96.3% (mean ±SD: 93.76 ± 1.98 %) for three serum samples being spiked with serum samples of different concentrations (Table 22). Observed-to-expected ratios for spiking recovery ranged from 92.9 to 105.9 % (mean ±SD: 98.2 ± 5.7 %) for four serum samples, using 122.0, 276.0, and 425.5 pg of cobalamin from the quality control sample provided by the manufacturer (Table 23). Intra-assay coefficient of variation (% CV) ranged from 0.4 - 3.4%. Inter-assay coefficient of variation (%CV) ranged from 3.5 - 8.4% (Table 24). The analytical sensitivity for this assay is reported to be 125 pg/mL by the manufacturer.

Table 20. Results for manually prepared dilutional parallelism of serum samples for serum cobalamin concentrations in the marmoset. Reprinted with permission.[119]

	Dilution	Observed (pg/mL)	Expected (pg/mL)	O/E (%)
Serum 1	0	485		
	1:2	283	242.5	116.7
	1:4	167	121.3*	N/A
	1:8	165	60.6*	N/A
Serum 2	0	391		
	1:2	262	195.5	134.0
	1:4	188	97.8*	N/A
	1:8	<150	48.9*	N/A
Serum 3	0	202		
	1:2	163	101.0*	N/A
	1:4	<150	50.5*	N/A
	1:8	<150	25.3*	N/A
Serum 4	0	456		
	1:2	258	228.0	113.2
	1:4	182	64.5*	N/A
	1:8	<150	57.0*	N/A

* indicates that the concentration is below the lower limit of detection of the assay.

Table 21. Results for automatically prepared dilutional parallelism for serum cobalamin concentrations in the marmoset. Reprinted with permission.[119]

Serum sample	Dilution used on the Immulite	Observed Vitamin B12 concentration (pg/mL)	Coefficient of variation (%) between samples
Serum 1	1:3	1580	5.8
	1:5	1392	
	1:10	1580	
	1:20	N/A	
Serum 2	1:3	1708	7.0
	1:5	1445	
	1:10	1644	
	1:20	N/A	
Serum 3	1:3	1923	8.8
	1:5	1728	
	1:10	1550	
	1:20	N/A	
Serum 4	1:3	1665	3.8
	1:5	1538	
	1:10	1674	
	1:20	N/A	

Table 22. Results for spiking recovery of serum samples with other serum samples with known serum cobalamin concentrations. Reprinted with permission.[119]

Spiking 1:1	Expected (pg/mL)	Observed (pg/mL)	O/E (%)
Serum 1: Serum 3	340.1	318.0	93.5
Serum 2: Serum 3	293.0	268.0	91.5
Serum 3: Serum 4	325.0	313.0	96.3

Cobalamin concentrations were 486.6pg/mL, 392.4pg/mL, 193.6pg/mL, and 456.4pg/mL for serum 1, serum 2, serum 3, and serum 4 respectively.

Table 23. Results for spiking recovery of serum samples using cobalamin Immulite standards (QC). Reprinted with permission.[119]

Serum sample	Concentration of spiking solution (pg/mL)	Observed* cobalamin concentration (pg/mL)	Expected* cobalamin concentration (pg/mL)	O/E %	O/E %
Serum 1	0 (diluent)	297			92.9
	244	374	419	89.3	
	552	513	573	89.5	
	851	722	722.5	99.9	
Serum 2	0 (diluent)	263			95
	244	356	385	92.5	
	552	502	539	93.1	
	851	685	688.5	99.5	
Serum 3	0 (diluent)	257			105.9
	244	378	379	99.7	
	552	551	533	103.4	
	851	783	682.5	114.7	

Table 23. Continued

Serum sample	Concentration of spiking solution (pg/mL)	Observed* cobalamin concentration (pg/mL)	Expected* cobalamin concentration (pg/mL)	O/E %	O/E %
Serum 4	0 (diluent)	278			99
	244	388	400	97	
	552	570	554	102.9	
	851	683	703.5	97.1	

*100µL of the spiking solution was added to 100µL of the serum samples.

Table 24: Precision and reproducibility for the measurement of serum cobalamin concentrations in the common marmoset using the Immulite cobalamin assay. Reprinted with permission.[119]

Sample	Number of repeats	Mean (pg/mL)	Standard deviation (pg/mL)	Coefficient of variation (%)
Intra-assay variability				
Serum 1	5	449.8	15.6	3.5
Serum 2	5	353.4	26.4	7.5
Serum 3	5	172.8	14.5	8.4
Serum 4	5	450.2	24.0	5.3
Inter-assay variability				
Serum 5	5	486.6	15.9	3.3
Serum 6	5	392.4	1.7	0.4
Serum 7	5	193.6	6.4	3.3
Serum 8	5	456.4	15.4	3.4

For the folate measurements: observed-to-expected ratios for 1:1, 1:2, 1:4, and 1:8 dilutions ranged from 63.0 to 95.0% (mean ±SD: 73.10 ±9.68%) (Table 25). The CV% for the concentrations of serum folate using dilutions available on the Immulite were 8.7%, 9.2%, 11.9%, 16.9%, and 20.6% for the 5 serum samples at four different dilutions of 1:10, 1:20, 1:40, and 1:100 (Table 26). Observed-to-expected ratios for spiking recovery ranged from 90.1 to 103.2% (mean ± SD: 96.99 ± 6.59%) for three serum samples being spiked with other serum samples with different concentrations (Table 27). Observed-to-expected ratios for spiking recovery ranged from 75.0 to 101.4 % (mean ±SD: 85.3 ± 4.6 %) for the five serum samples, using 1.5, 7.0, and 10.9 ng of folate from the quality control sample provided by the manufacturer (Table 28). The intra-assay coefficient of variation (%CV) ranged from 1.8 - 9.4%. Inter-assay coefficient of variation (%CV) ranged from 3.7 - 5.9% (Table 29). The analytical sensitivity for this assay is 0.8 ng/mL as reported by the manufacturer.

No statistically significant differences in cobalamin and folate concentrations in six serum samples stored at -80^{o}C between 2006 and 2013 were identified (P = 0.58, P = 0.81 respectively). The median [minimum – maximum] CV% for the cobalamin and folate concentrations for the six serum samples for the measurements made in 2006 and 2013 were 10.5% [2.9 - 21.9%], and 12.5% [2.5 - 22.8%].

6.3.2 Reference intervals

Serum samples from a total of 53 marmosets were available, 34 of which were from the BMAC and 19 from the SNPRC. Of these animals, 14 marmosets (BMAC 8, SNPRC 6) had a history of clinical signs associated with gastrointestinal disease, and were excluded from

Table 25. Results for manually prepared dilutional parallelism of serum samples for serum folate concentrations in the marmoset. Reprinted with permission.[119]

Dilution	Observed (O) ng/mL	Expected (E) ng/mL	O/E (%)
Serum 1			
1:1	13.1		
1:2	5.4	6.6	82.3
1:4	2.1	3.3	63.8
1:8	1.1	1.6	64.7
Serum 2			
1:1	17.9		
1:2	7	9	77.9
1:4	2.9	4.5	64.8
1:8	1.4	2.2	63
Serum 3			
1:1	9.6		
1:2	3.4	4.8	70.6
1:4	1.6	2.4	66.9
1:8	<1.0*	1.2	

Table 25. Continued

Dilution	Observed (O) ng/mL	Expected (E) ng/mL	O/E (%)
Serum 4			
1:1	20.6		
1:2	9.8	10.3	95
1:4	4.2	5.2	81.7
1:8	1.9	2.6	73.4

* indicates that the concentration is below the lower limit of detection of the assay.

Table 26. Results for automatically prepared dilutional parallelism for serum folate concentrations in the marmoset. Reprinted with permission.[119]

Serum sample	Dilution used on the Immulite	Observed folate concentration (ng/mL)	Coefficient of variation (%) between samples
Serum 1	1:10	-	8.7
	1:20	-	
	1:40	629	
	0.11111	556	
Serum 2	1:10	209	20.6
	1:20	179	
	1:40	160	
	1:100	126	
Serum 3	1:10	-	11.9
	1:20	-	
	1:40	488	
	1:100	412	

Table 26. Continued

Serum sample	Dilution used on the Immulite	Observed folate concentration (ng/mL)	Coefficient of variation (%) between samples
Serum 4	1:10	-	9.2
	1:20	322	
	1:40	316	
	1:100	271	
Serum 5	1:10	-	16.9
	1:20	-	
	1:40	675	
	1:100	690	

Table 27. Results for spiking recovery of serum samples with other serum samples with known serum folate concentrations. Reprinted with permission.[119]

Spiking 1:1	Expected (E) folate concentration (ng/mL)	Observed (O) folate concentration (ng/mL)	O/E (%)
Serum 1: Serum 2	11.4	11.1	97.7
Serum 1: Serum 3	13.8	12.4	90.1
Serum 1: Serum 4	15.1	15.6	103.2

Folate concentrations were 9.6 ng/mL, 13.1 ng/mL, 17.9 ng/mL, and 20.6 ng/mL for serum 1, serum 2, serum 3, and serum 4 respectively.

Table 28. Results for spiking recovery of serum samples using folate Immulite standards (QC). Reprinted with permission.[119]

Serum sample	Concentration added (ng)	Observed folate concentration (ng/mL)	Expected folate concentration (ng/mL)	O/E %	O/E%
Serum 1	-	6.3			
	1.5	7.7	7.8	98.2	
	7.0	9.4	13.3	70.7	82.2
	10.9	13.4	17.2	77.8	
Serum 2	-	1.2			
	1.5	2.9	2.7	109.6	
	7.0	6.0	8.2	72.9	86.1
	10.9	9.1	12.1	75.7	
Serum 3	-	5.4	5.4		
	1.5	6.5	7.0	93.2	
	7.0	9.7	12.5	77.6	81.8
	10.9	12.2	16.3	74.7	

Table 28. Continued

Serum sample	Concentration added (ng)	Observed folate concentration (ng/mL)	Expected folate concentration (ng/mL)	O/E %	O/E%
Serum 4	-	3.2			
	1.5	3.9	4.7	82.6	
	7.0	7.0	10.2	67.9	75.0
	10.9	10.5	14.1	74.3	
Serum 5	-	4.2			
	1.5	3.8	5.7	66.9	
	7.0	14.5	11.2	129.3	101.4
	10.9	16.3	15.1	107.9	

*100µL of the spiking sample was added to 100µL of the serum samples.

Table 29. Precision and reproducibility for the measurement of serum folate concentrations in the common marmoset using the Immulite folate assay. Reprinted with permission.[119]

Sample	Number of repeats	Mean (ng/ml)	Standard Deviation (ng/ml)	Coefficient of variation (%)
Intra-assay variability				
Serum 1	5	13.8	0.5	3.7
Serum 2	5	18.2	1.7	9.4
Serum 3	5	9.7	0.5	5.4
Serum 4	5	22.2	0.4	1.8
Inter-assay variability				
Serum 5	5	13.9	0.8	6.0
Serum 6	5	16.8	0.9	5.5
Serum 7	5	9.6	0.4	3.7
Serum 8	5	21.8	0.9	4.1

calculating the reference interval. Reasons for exclusion included weight loss (7), a diagnosis of Giardiasis or treatment for Giardia (6), loose stool (1), and a transient gastrointestinal upset (1).

Due to lack of sufficient sample volume for down dilution, a final concentration was not determined for samples from some animals. A reference interval for serum cobalamin was established as 322 – 2,642 pg/mL using samples form 35 healthy marmosets. In order to achieve the final cobalamin concentrations, serum samples were run without any dilution (neat) for 18 samples and in a 1:3 dilution for 17 marmosets samples, respectively.

A reference interval for serum folate concentrations was established as 54.8 – 786.4 ng/mL using 37 samples form healthy marmosets. Twenty nine samples were run in 1:40 dilution, 5 in a 1:20, 2 in a 1:100 dilutions, and 1 without any dilution to achieve the final concentration.

6.3.3 Health comparisons

Gender, dam, sire, birthplace, multiple births, litter size at weaning, obesity status, and age group were not significantly associated with either serum cobalamin or serum folate concentration (Table 30). Interestingly, serum folate concentrations were significantly different between the two centers (BMAC, SNPRC) where the samples had been collected (P=0.0108).

Serum cobalamin concentrations were significantly different (P=0.0002) between the healthy marmosets (median (range): 1,020 pg/mL (281-2,390 pg/mL)) and the 14 marmosets with a previous history of gastrointestinal disease (median (range): 536 pg/mL (150-1,051 pg/mL)). However, there was no significant difference folate serum concentrations between the two groups (P=0.356).

Table 30. Table showing the different factors, the respective P values, and adjusted P values for serum cobalamin and folate concentrations. Reprinted with permission.[119]

Factors	Folate		Cobalamin	
	P value	Adjusted P values	P value	Adjusted P values
Source (SNPRC, BMAC)	0.001*	**0.011***	0.289	2.603
Sex	0.525	4.725	0.574	5.170
Dam	0.088	0.794	0.128	1.150
Sire	0.126	1.134	0.069	0.617
Birthplace	0.092	0.825	0.066	0.594
Multiple Births	0.080	0.716	0.263	2.363
Weaned Litter Size	0.682	6.138	0.418	3.761
Obesity Status	0.273	2.455	0.325	2.923
Age Group	0.477	4.289	0.141	1.265

6.3.4 Repeated measurements of serum cobalamin and folate

Repeated measurements of serum cobalamin and folate from the same animals were available from at least two different time points for 30 animals, with 8 animals having samples from 3 different time points, and 1 animal having samples from 4 different time points. Due to the lack of sufficient volume, cobalamin measurements could not be performed in 6 serum samples, and folate measurements could not be performed in 2 serum samples. In 23 animals, repeat serum samples for cobalamin concentration were available after a median of 120 days [minimum – maximum: 77 -165] of the first collection and results were not significantly different from baseline measurement (p= 0.726). In 8 animals, serum samples for cobalamin concentration measurements were available after a median of 223 days [minimum – maximum: 222 - 258] and were also not significantly different from baseline measurements (P= 0.547).

In 26 animals, repeat serum samples for the measurement of folate concentration were available after a median of 123 days [minimum – maximum: 88 -165] of the first collection and results were not significantly different from the first measurement (P= 1). In 7 animals, serum samples for the measurement of folate concentration were available after a median of 223 days [minimum – maximum: 222 - 258] after baseline collection and were also not significantly different (P = 0.375).

6.3.5 Serum cobalamin and folate concentrations in marmosets with CLE and gastrointestinal disease

Serum cobalamin and folate concentrations and paired necropsy diagnosis was available for 38 marmosets from the NEPRC. Of these, 8 marmosets were healthy, 11 marmosets had CLE, and 18 marmosets died or were euthanized because of other diseases including: intestinal

adenocarcinoma (2), chronic nephritis / renal disease (15), hepatic lipidosis (4), pancreatitis (2), peritonitis (1), cholecystitis (4), steatohepatitis (1), chronic prostatitis (1), enteropathogenic *E. coli* (1), lymphoma and ulcerative enteritis (1). Median [minimum – maximum] serum cobalamin concentrations were 226 pg/mL [213- 627 pg/mL] in healthy marmosets, 225 pg/mL [149 – 514 pg/mL] in marmosets with CLE, and 297 pg/mL [149 – 663 pg/mL] in marmosets with other diseases and serum cobalamin concentrations were not significantly different between the 3 groups (P= 0.148). Median [minimum – maximum] serum folate concentrations were median [minimum – maximum] 141 ng/mL [84- 230 ng/mL] in healthy marmosets, 68.6 ng/mL [18.3 – 226 ng/mL] in marmosets with CLE, and 203 ng/mL [28.3 – 327 ng/mL] in marmosets with other diseases respectively and serum folate concentrations were significantly different between the 3 groups (P= 0.001). Furthermore, serum folate concentrations were significantly different between marmosets with CLE and those with other diseases (P= 0.008).

Sensitivity and specificity for low serum cobalamin concentrations using a cutoff value of <322.0 pg/mL for the diagnosis CLE were 44.4% (95% CI; 21.5% to 69.2%) and 81.8% (95% CI: 48.2% to 97.7%), respectively. Sensitivity and specificity for low serum folate concentrations using a cutoff value of <54.8 ng/mL for the diagnosis of CLE were 71.4% (95% CI: 29.0% to 96.3%) and 73.9% (95% CI: 51.69% to 89.8%), respectively.

Given that the other diseases, such as *E.coli* infection or intestinal carcinoma involved the gastrointestinal tract, an attempt was further made to classify the animals as healthy (n=8), those with diseases of the gastrointestinal tract (n=15), and those with no diseases of the gastrointestinal tract (n=15). Serum cobalamin concentrations were not significantly different between the 3 groups (P= 0.484). However, serum folate concentrations were significantly different between the

3 groups (P= 0.040). Furthermore, serum folate concentrations were significantly different between marmosets with GI disease and those with extra-gastrointestinal diseases (P= 0.036). Sensitivity and specificity for a low serum cobalamin concentration using a <322.0 pg/mL cutoff value detection of GI disease were 57.9% (95% CI; 33.5% to 79.8%) and 63.6% (95% CI: 30.8% to 89.1%), respectively. Sensitivity and specificity for a low serum folate concentration using a <54.8 ng/mL as a cut off for gastrointestinal disease were 85.7% (95% CI: 42.1% to 99.6%) and 60.9% (95% CI: 35.5% to 80.3%), respectively.

6.3.6 Cross sections and longitudinal measurements of serum cobalamin and folate concentrations

In a cross-sectional study serum cobalamin and folate concentrations were measured in 43 animals from the NEPRC. Using a cobalamin concentration of <322.0 pg/mL as a cut-off value for a low cobalamin, 15/43 animals had a decreased serum cobalamin concentration. Using a serum folate concentration of <54.8 ng/mL as a cut-off value for a low folate, 2/43 animals had a decreased serum folate concentration.

In a longitudinal study, serum cobalamin and folate concentrations were measured in 4 animals from the NEPRC over a period of several years. Final necropsy diagnosis, cobalamin, and folate concentrations were also available for review (Table 31). Serum cobalamin and folate concentrations for these 4 animals are shown in Figures 8 and 9, respectively.

6.4 Discussion

Analytical validation of an assay is neccessary when immunoassays are used for the first time in any species. Species specific reference intervals also need to be generated and often a the dilution of samples to bring them into the working range of an assay is required.[146] We performed

Table 31. Serum cobalamin and serum folate concentrations over time as well as necropsy findings in the 4 marmosets from the NEPRC (longitudinal study). Reprinted with permission.[119]

Marmoset	Necropsy findings	Days*	Cobalamin concentration** (pg/mL)			Folate concentration*** (ng/mL)		
			Median	Minimum	Maximum	Median	Minimum	Maximum
1	CLE, nephritis	1775	>1001	882	>1001	156	104	772
2	CLE, cholecystitis, and nephritis	3514	391	391	>1001	106	15.9	529
3	Pancreatitis, hepatic amyloidosis, and cholecystitis	2283	224	149	362	158.5	103	300
4	No significant findings	2654	<149	<149	213	250	33.1	786

CLE = chronic lymphocytic enteritis

* Days between the first and last sample available for testing.

** See Figure 8 for display of serum cobalamin concentrations over time

*** See Figure 9 for display of serum folate concentrations over time

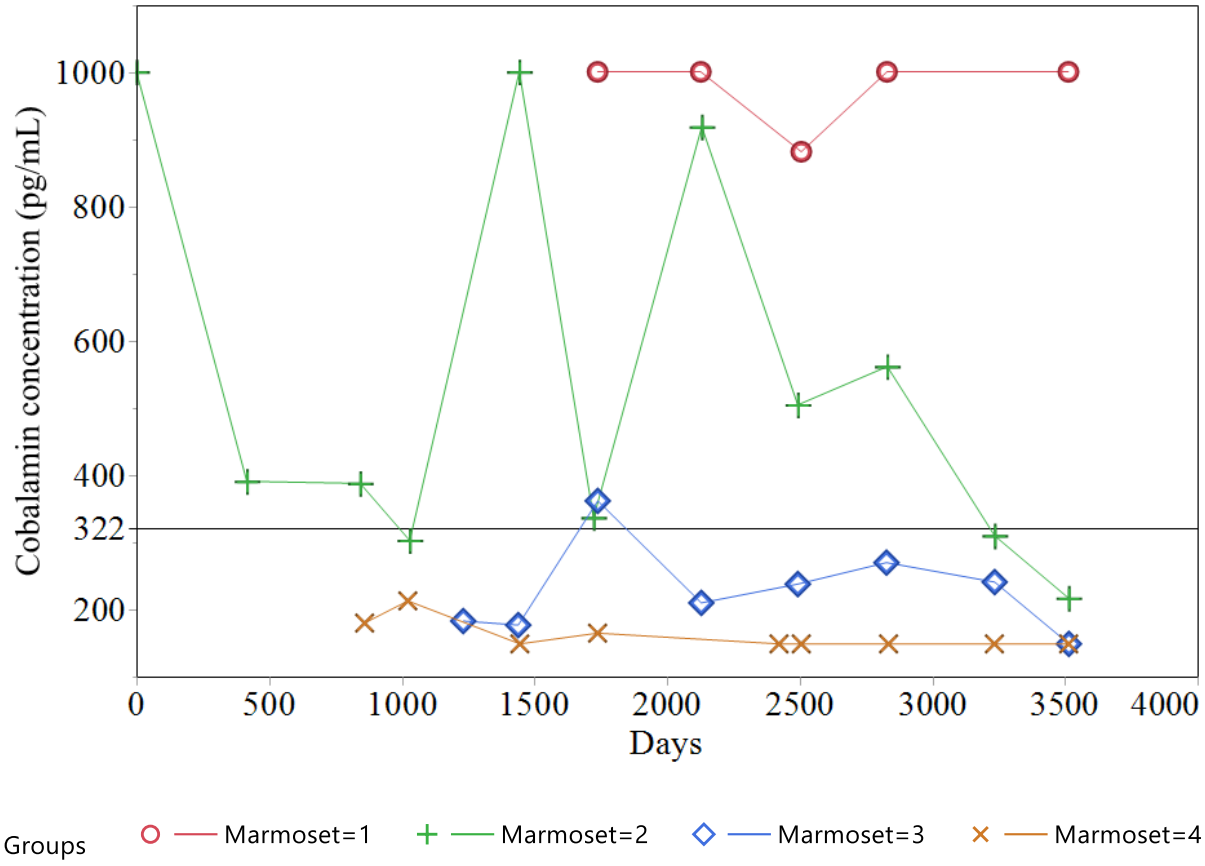

Figure 8. Serum cobalamin concentrations in 4 marmosets at the NEPRC over a period of several years. Reprinted with permission.[119]

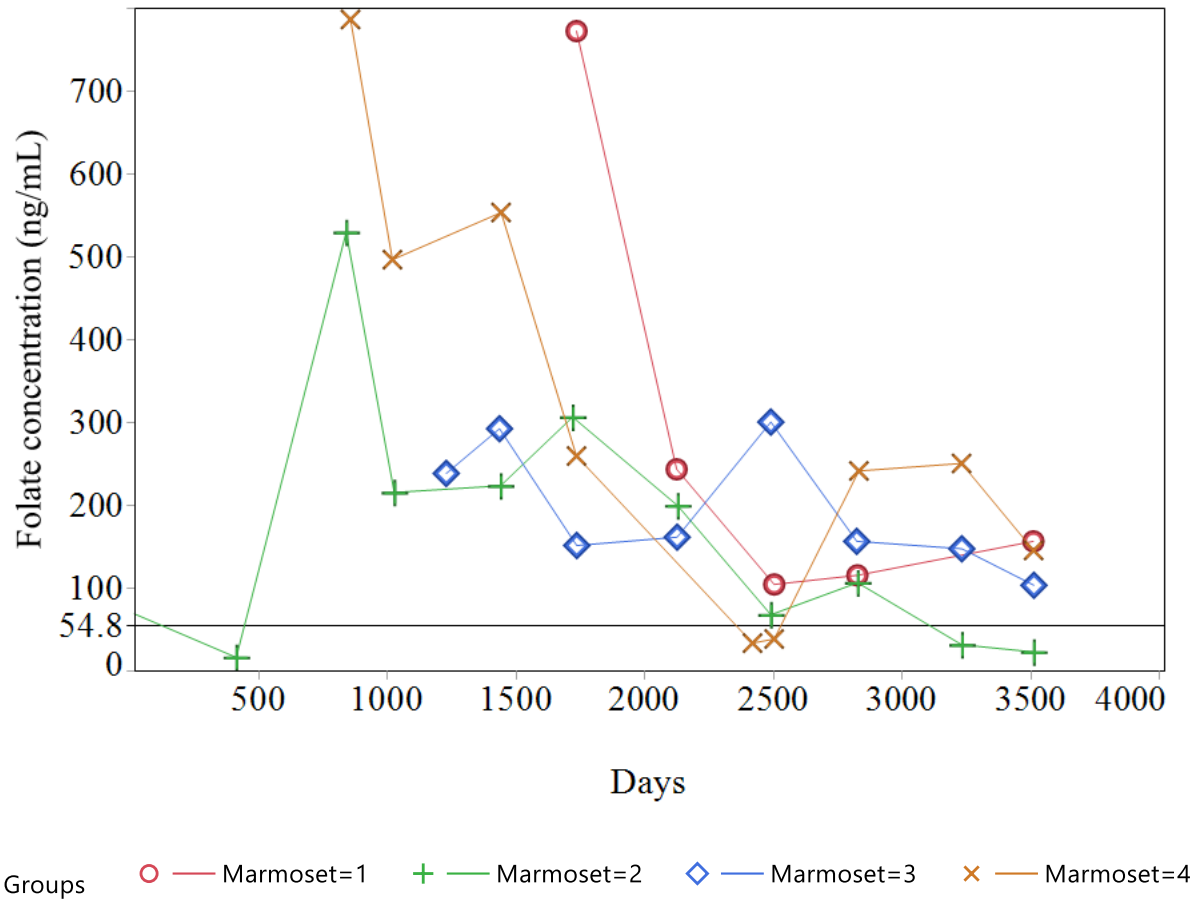

Figure 9. Serum folate concentrations in 4 marmosets at the NEPRC over a period of several years. Reprinted with permission.[119]

a partial analytical validation of commercially available assays for the measurement of serum cobalamin and folate concentrations in humans for use in the common marmoset. The majority of the validation study was performed using pooled serum samples because of the lack of sufficient sample volumes from using samples from individual marmosets. This makes the detection of a possible matrix effect difficult. However, we showed that the Immulite assay for cobalamin and folate measurement is linear, accurate, precise, and reproducible for use with serum samples from the common marmosets. In the assessment of linearity by manual two-fold dilution, the samples chosen for cobalamin and folate were in the lower end of the working range of the assay, hence the increased O/E ratios. However, when samples with higher concentrations were diluted using the dilution function available within the Immulite, their CV% were within the accepted value of <30% for immunoassays,[41] and very close to the stringent criteria of <20%,[122] with only one value being 20.6%.

The use of banked samples did not affect the concentration measurements made in this study. At least from our limited data set it would appear that serum cobalamin and folate concentrations are relatively stable when serum samples are stored at -80^{o}C for up to 7 years.

The reference interval for cobalamin established in the common marmoset (322 – 2,642 pg/mL) is comparable to that reported in pig-tailed macaques (961 to 2,000 pg/mL) and rhesus macaques (727 to 2,000 pg/mL), using the same assay.[65] Also, in vervet monkeys (*Cercopithecus aethiops*) using two different radioimmunoassays, plasma vitamin B12 concentrations have been reported in a similar interval; 1,545 ±451 pg/mL and 1,396 ± 450 pg/mL.[162]

Serum folate concentrations have been reported in the Rhesus macaque using a microbiological assay using *L. casei* [103] for measurement and in the plasma of vervet monkeys using two immunoassays and were 15.0 ± 2.4 ng/ml , 10.2 ± 3.7 ng/mL , and 8.2 ± 2.2 ng/mL,

respectively. This is very different from the reference interval established in the common marmoset in this study and is probably related to the methodology employed. A dietary component cannot be ruled out as New World primate diets contain a folate supplement and the folate concentration seen in our study is significantly higher than what is observed in other monogastric species.

Serum cobalamin concentrations in marmosets were not significantly different between the two primate centers (SNPRC and BMAC). It is possible that cobalamin concentrations might have been more diagnostic for CLE if the marmoset diets were not supplemented. In the wild, insects and small mammals serve as sources of cobalamin and comparing cobalamin concentrations between marmosets in the wild and those in primate research colonies may provide additional insights into the role of cobalamin and CLE.

However, serum folate concentrations were significantly different between the two centers. Marmosets from the SNPRC did have higher serum folate concentrations than those at the BMAC. This could be attributed to three possible reasons, a dietary component, a diet treatment component, or differences in the gut microbiota. Marmosets at the BMAC were fed a purified diet (#TD.07148 Marmoset Diet, Harlan Teklad Madison, WI), and a primate enrichment mixture consisting of nuts, seeds, and dried fruit from the same manufacturer. However, both these diet components are also irradiated at the manufacturing plant. Marmosets at the SNPRC are on two diets, a commercial New World primate diet (Mazuri diet (AVP Callitrichid 5LK6) and also on the same purified diet (without irradiation) used at the BMAC. Diet for marmosets at the SNPRC also included daily food enrichment, such as yogurt, fruits, cheerios, etc. which are also good additional sources of folate.[40] Supplementation of folate in the diet does lead to increased plasma concentrations in other primate species.[162] Irradiation has been reported to decrease folic acid

concentrations by 20-30% in certain foods[162] and it is not known if irradiation had a similar effect here. Folate is mainly synthesized by jejunal bacteria[77] and differences in the fecal microbiome have been reported between the marmosets of these two colonies,[130] and this could be another reason for changes in serum folate concentrations between the two centers. Given these observations, marmoset colony specific reference intervals for serum folate concentrations perhaps may be more appropriate specifically for colony monitoring purposes for gastrointestinal health.

While folate concentrations were not significantly different between marmosets with a history of gastrointestinal disease and those without such a history, serum cobalamin concentration was. However, only 3/14 of the marmosets (21.4%) had serum cobalamin concentrations, lower than the established reference interval. In a recent study,[65] looking at macaques with chronic diarrhea, 5/6 pigtail macaques had decreased serum cobalamin concentrations, but all 6 rhesus macaques with chronic diarrhea had normal cobalamin concentrations. Cobalamin concentrations are reported to be low in some humans with IBD,[10] and dogs with chronic enteropathy.[14] A dietary cause for a decreased serum cobalamin concentration is unlikely in these marmosets fed a commercial diet.[65] Rhesus macaques fed cobalamin deficient diets were asymptomatic for 12-18 months despite severely decreased serum cobalamin concentrations.[73] Therefore, absence of clinical signs are not sufficient to assume normal serum cobalamin concentrations. Other reasons for low cobalamin concentrations include genetic defects, changes in the gut microbiota,[140] hyperthyroidism,[34] exocrine pancreatic insufficiency, [141] lymphoma,[141] ageing,[167] and metabolic causes.[145]

The diagnostic utility of decreased serum concentrations of cobalamin and/or folate for the diagnosis of CLE or gastrointestinal disease in the common marmoset, were suboptimal; lower serum folate concentrations were moderately sensitive (>70%) for gastrointestinal disease or CLE,

and lower cobalamin concentrations were specific (>80%) for CLE. This makes their utility in the diagnosis of CLE or occult gastrointestinal disease limited. Nonetheless, it may serve as a screening test as no ante-mortem tests are currently available. However, it does raise questions regarding the value of supplementation of these vitamins in animals with CLE or gastrointestinal disease. False positive and false negative rates for detecting cobalamin deficiency using commercial assays are as high as 50% in human beings.[145] This is because these assays measure intrinsic factor binding of cobalamin, and measures both serum holohaptocorrin and serum holotranscobalamin and can mask true deficiency or falsely imply a deficient state. For this reason, in human beings, a low cobalamin concentration triggers the measurement of a functional biomarker such as methylmalonic acid and/or homocysteine, which are considered confirmatory.[29,42] Studies in dogs with chronic gastrointestinal disease have also shown that only a subset of dogs do have decreased cobalamin concentrations, and only a subset of these dogs have increased serum methylmalonic acid concentrations, suggesting a deficiency of cobalamin at the cellular level.[14,16] Thus, additional studies are warranted in the common marmoset, to evaluate cobalamin and folate deficiencies at the cellular level.

Lower folate concentrations in marmosets with CLE and gastrointestinal disease are interesting as marmosets raised on a folate restricted diet developed clinical signs that terminated fatally in 59 to 136 days.[43] These clinical signs observed included anorexia, weight loss, diarrhea, alopecia, ulceration of the oral and intestinal mucosa, anemia, leucopenia and granulocytopenia, some of which have been reported with marmosets with CLE. Serum folate measurements, when compared to cobalamin concentrations, are adequate for the diagnosis of folate deficiency and red blood cell folate measurement are only indicated rarely.[48]

The fact that serum concentrations of cobalamin and folate from the marmosets from the same colony measured 120 and 220 days apart did not change significantly would suggest that the concentrations are stable over relatively long periods of time. The data from the 4 animals from the NEPRC show that normal marmosets can have very low cobalamin concentrations for long periods of time without any clinical signs (marmoset 4), and marmosets with CLE can have normal serum cobalamin and folate concentrations (marmoset 1). It is noteworthy, that in one animal with CLE, cobalamin and folate concentrations (marmoset 2) did decrease in over 600 days. It remains unknown if there are subsets of marmosets with CLE based on their response to diets, antibiotics, or immunosuppressive (steroids) drugs, as has been described for other species, such as the dog.[70] The major limitation of this study was that it was based on archived samples. Blood volumes from the marmosets were a limiting factor and pooled samples were used for the validation, which was not ideal as it did not allow us to fully assess the presence of any matrix effects. Also, a relatively small number of animals were used to establish the reference intervals. In an ideal scenario serum samples from at least 120 marmosets would have been used to establish the reference intervals as recommended by the International Federation of Clinical Chemistry (IFCC).

To conclude, we validated a commercially available assay for the measurement of serum cobalamin and folate for use in humans in the common marmoset. We also established reference intervals for serum cobalamin and folate concentrations in the common marmoset. The difference in serum folate concentration between centers suggests that facility specific reference intervals may be more appropriate for this analyte. Low serum cobalamin and folate concentrations were observed in some common marmosets with gastrointestinal disease, and chronic lymphocytic enteritis. However, additional studies are necessary to further elucidate the utility of these measurements in the common marmoset.

CHAPTER VII

FECAL N-METHYLHISTAMINE CONCENTRATIONS IN THE COMMON MARMOSET (*CALLITHRIX JACCHUS*). ∞∞

7.1 Introduction

The common marmoset (*Callithrix jacchus*) is a New World monkey employed in biomedical research since the early 1960's. Its popularity has increased over the years,[1] particularly because of their small size, reduced cost for maintenance compared to larger primates, easy husbandry, rapid reproductive turnover, and decreased susceptibility to certain human pathogens.[90] Inflammatory diseases of the gastrointestinal tract, particularly of the large intestine, have been described in marmosets since their initial use in biomedical research.[32,38,158] Inflammatory bowel disease (IBD), particularly chronic lymphocytic enteritis (CLE), has been a consistent finding in *C. jacchus* colonies with prevalence rates as high as 60.5% of all marmosets used as controls in various studies at one center.[88] No specific etiology has been identified; however many etiological factors such as, gluten sensitivity, dietary protein deficiency, and the pancreatic spirurid nematode (*Trichospirura leptostoma*) have been suspected. Clinically, failure to thrive in juveniles, or weight loss in adults, with or without diarrhea are observed. Diffuse to segmental lymphocytic enteritis is seen on histology.[88]

Currently an antemortem diagnosis is made based on clinical signs, a history of weight loss, and a decreased serum albumin concentration.[11] No proven effective treatment exists and a final diagnosis is usually[112] made only at necropsy. Newer markers like fecal calprotectin,[108] fecal

∞∞Submitted to Comparative Medicine. Parambeth JC, López RF, Lopes R, Keyser S, J Lidbury JA, Suchodolski JS, Steiner JM. Fecal N-methylhistamine concentrations in the common marmoset (*Callithrix jacchus*).

α_1-PI, serum matrix metalloproteinases,[171] serum IgA antibodies to gliadin and related proteins[84] are being investigated and may serve as markers in the future. Treatment with steroids,[115] or tranexamic acid [172] may also hold some promise in the future.

Mast cells have been described in the gastrointestinal tracts of dogs[50] and humans,[127] and have been investigated in chronic enteropathies of dogs[6,13] and inflammatory bowel disease[111] in humans.

The role of mast cells has not been determined in marmoset CLE. Mast cells can be missed on routine hematoxylin-eosin staining. Their detection requires either special staining with toluidine blue or immunohistochemistry for mast cell tryptase.[6,13] Markers of mast cell activation rather than their absolute numbers seen on histology have also been suggested as a better method to measure their activity. Histamine is primarily stored in the mast cells and serves as marker of mast cell degranulation. Rapid metabolism of released histamine leads to N-methylhistamine (NMH) and imidazole acetaldehyde.[153] Fecal concentrations of NMH were found to be increased in a subset of dogs with chronic enteropathies, suggesting that mast cell mediated inflammation plays a pathogenic role.[6,13]

We hypothesize that fecal NMH concentrations are increased in marmosets with chronic lymphocytic enteritis and this could be used as a marker of intestinal inflammation in these patients. Therefore, the aim of this study was to measure NMH concentrations in fecal samples collected from healthy marmosets, establish a reference interval, and compare the concentrations from healthy marmosets to marmosets with chronic lymphocytic enteritis.

7.2 Materials and methods

7.2.1 Sample collection

Samples were collected from a total of 30 healthy marmosets maintained at the Southwest National Primate Research Center (SNPRC), Texas Biomedical Research Institute, San Antonio, Texas and the Barshop Marmoset Aging Center (BMAC), The University of Texas Health Science Center, San Antonio, Texas. Collection of fecal samples was approved from both institutional IUCAC (AUP # 1259CJ for Texas Biomed, and 06120X for UTHSCSA). Three consecutive, naturally passed fecal samples were collected from each marmoset in a fecal collection tube (Fecal collection tube (101 × 16.5 mm; with spatula), Sarstedt AG&Co., Nümbrecht, Germany) and frozen immediately. Marmosets sampled did not have had any clinical signs of gastrointestinal disease and were not part of any research studies during the sampling process.

Single time point fecal samples were also collected per rectally from 16 marmosets at necropsy. These marmosets died or were euthanized at the New England Primate Research Center (NEPRC), Southborough, MA; *t*hese samples were frozen immediately after collection. The necropsy and sample banking were part of routine colony management procedures and included sick and healthy marmosets. The investigators were blinded to the final postmortem diagnosis of these animals when the assays were run. Gastrointestinal tissue samples where available were also obtained from these animals for additional staining for mast cells.

All the fecal samples were shipped to the Gastrointestinal Laboratory at Texas A&M University overnight with dry ice. Fecal samples were stored at -80^{o}C and extracted later. Fecal extracts were stored at -80^{o}C until NMH analysis.

7.2.2 Preparation of fecal samples.

A fecal NMH extraction protocol similar to that previously described in domestic dogs [133] was used. Briefly, fecal samples were extracted in 1:5 phosphate buffered saline *(*BupH™ Phosphate buffered Saline, Thermo Scientific, Rockford, IL)-new born calf serum buffer (Newborn Calf Serum, Sigma-Aldrich Co., St. Louis, MO), followed by vigorous shaking in an automated shaker for 20 minutes. The fecal tubes were then centrifuged at 3,000g for 20 minutes and filtered using a serum filter (Fisherbrand® serum filter system, Fisher Scientific Inc., Pittsburgh, PA). The resultant supernatant, (fecal extract) was used for the assay.

7.2.3 N-methylhistamine assay

N-methylhistamine in the fecal extracts was measured using stable isotope dilution gas chromatography/mass spectrometry (GC/MS) as previously described for those from domestic dogs.[133] Briefly, 50 pg of trideuterated NMH *(*CDN Isotopes, Pointe Claire, QC, Canada*)* was added to 200µL of fecal extract as an internal standard. Then 200µL of borate buffer (pH 9, 10mM) was added. The sample was vortexed and then applied on a solid phase silica extraction column (Sep-Pak cartridge, Waters, Milford, Massachusetts). The columns were washed with changes of chromatography grade water and then the sample was eluted with 0.1N HCl acidified methanol (VWR International, West Chester, Pennsylvania). The eluted samples were evaporated to dryness using nitrogen on a heating block. The dried sample was reconstituted with 300 µL of 20% methanol in chloroform (VWR International, West Chester, Pennsylvania), before application to the second solid phase silica extraction column. The column was washed with 150µl of 20% methanol in chloroform (VWR International, West Chester, Pennsylvania). The sample was eluted

with four one mL volumes of methanol:chloroform:ammonium hydroxide (25:25:1, v/v) and dried as before. Derivitization was achieved by adding 200µl ethyl acetate (Sigma-Aldrich Corp., St. Louis, MO, USA), 40µl pyridine (Thermo Fisher Scientific, 168 Third Avenue, Waltham, Massachusetts), and 100µl pentafluoropropionic anhydride (Sigma-Aldrich Corp., St. Louis, MO, USA), to the sample and incubating at ~64ºC for 40 minutes. The samples were evaporated to complete dryness as before. In the next partitioning step, 500µL of 0.5 M Tris buffer was added to each sample, followed by 1.5 ml of hexane (Sigma-Aldrich Corp., St. Louis, MO, USA). The samples were vortexed for 1 minute and centrifuged at 574 g for one minute. The hexane top layer was collected and another 1.5 mL of hexane was added to each sample and the process was repeated. The two hexane fractions were combined and evaporated to dryness Before transferring to a GC/MS autosampler vial, the residue was reconstituted with 30 µL of ethyl acetate and vortexed. The GC/MS analysis was performed using the Agilent 690N GC and 5975C MSD with a dimethylpolysiloxance capillary column, all other conditions (temperature, carrier gas, gradient, and pressure) used were similar to what has been described in the earlier fecal NMH assay in dogs. A standard curve from 0 to 5,000 pg/µL was run prior to each run to evaluate assay performance. NMH and deuterated isotopes were quantified by use of the ions at an m/z of 417 and 420, respectively. Fecal concentrations of NMH were back calculated for the wet weight of the sample and reported in ng/g feces.

7.2.4 Partial validation

Since the assay has not been validated for use in the common marmoset, a partial validation to assess linearity and accuracy was done. Linearity was determined by calculating observed-to-expected (O/E) ratios for three pooled (due to lack of sufficient volume) fecal extracts serially

diluted 1:2 and 1:4. Accuracy was measured by calculating O/E ratios for three pooled fecal extracts that were spiked with three different NMH concentrations (125 pg/µL, 500 pg/µL, and 1250 pg/µL; equivalent to assay standards).

7.2.5 Establishment of the reference interval in healthy marmosets

Single time point, 3-day mean and 3-day maximum concentrations for fecal NMH were measured and used to establish the reference interval for healthy marmosets. The coefficient of variation was calculated between the three-day collections. A reference interval was established by calculating the upper 95th percentiles of the single time point, the three-day mean, and the three-day maximum fecal NMH concentrations. For the single time point the sample from the 1st day of collection was utilized.

7.2.6 Necropsy results & fecal NMH concentrations

Full necropsy data, including gross findings, microscopic findings, and summary from the pathologist was obtained from 16 marmosets from the NEPRC who had fecal samples collected and analyzed. Fecal NMH concentrations were correlated to the postmortem findings.

7.2.7 Histology and mast cell counts

Gastrointestinal (stomach, small intestine and large intestine) sections collected at necropsy were obtained from the NEPRC where available and were stained with routine hematoxylin and eosin and toluidine blue using routine protocols. Sections stained with H&E were not revaluated but were assessed to ensure that they were full thickness sections and contained mucosa, submucosa and muscularis propria. Only full thickness sections were considered for the

toluidine blue staining. Sections stained with toluidine blue were evaluated by one board-certified anatomic pathologist (Lopez) and mast cells were counted in 4 high-power fields (400×) per tissue.

7.2.8 Statistical analysis

Statistical computation was performed with a commercial software package GraphPad PRISM5.0 (GraphPad software, Inc. La Jolla, CA) and statistical significance was set at $P<0.05$. In addition, the coefficient of variation (%CV = [standard deviation/mean] × 100) was used to determine the variability in the fecal concentrations of NMH from the three-day samples. Sensitivity and specificity will be calculated using an online calculator (https://www.medcalc.org/calc/diagnostic_test.php).

7.3 Results

For the partial validation of the assay, one of the three samples failed to go through the column as expected and had to be excluded. For assessment of the linearity, the O/E % was 137.1%, and 124.6% for one sample at 1:2, and 1:4 , respectively, and 101.4%, and 76.3% for the other sample at the same dilutions. Accuracy O/E % for the two fecal extracts that were spiked with three different NMH concentrations (125 pg/µL, 500 pg/µL, and 1250 pg/µL; equivalent to assay standards) were 84.5%, 85.2%, and 76.1% for one sample, and 74.1%, 80.7%, and 72.8% for the other.

Thirty healthy marmosets were used to establish the reference interval. Of these, 16 animals were from the SNPRC, and 14 were from the BMAC. The concentrations of NMH detected in fecal extracts ranged from undetectable to 216.1µg/g of feces. Using the upper 95^{th} percentile the reference intervals were determined to be ≤ 118.2ng/g of feces for a single fecal sample, ≤

121.7ng/g of feces for the 3-day mean, and ≤167.5ng/g for the 3-day maximum. The median inter-day coefficient of variation from each subject was 42.2% [minimum-maximum: 7.1- 141.4%].

Only 14 of the 16 single time point fecal samples collected from marmosets undergoing postmortem examination at the NEPRC were available for analysis. The particulars of the animals and the fecal NMH concentrations are shown in Table 32. Conditions included CLE (7), healthy controls (2), renal failure (2), lymphoma with ulcerative enteritis (1), enteropathogenic E. Coli (1), and adenocarcinoma (1). Diagnosis of CLE was established histologically. Seven of the 14 animals

Table 32. Summary of data in 14 marmosets from the NEPRC with fecal NMH concentrations. Reprinted with permission.[118]

Marmoset Number	Sex	Body Weight (g)	Age in days	Cause of death	Clinical history	Post mortem diagnosis based on gross and microscopic examination	Fecal NMH (μg/g)
1	Female	415.7	1243	Euthanized	Control for clinical study	Normal animal	18.0
2	Male	456.8	1400	Euthanized	Control for clinical study	Normal animal	35.0
3	Female	336.7	3757	Euthanized	Suspected CLE, wasting and poor health	Chronic lymphocytic enteritis	994.8
4	Male	398.9	Not available	Spontaneous found dead	None	Chronic lymphocytic enteritis	396.6
5	Male	292.8	5372	Euthanized	Suspect mass in abdomen	Chronic lymphocytic enteritis	219.5
6	Female	485.5	Not available	Euthanized	Diabetic with lymphocytosis	Chronic lymphocytic enteritis	228.6
7	Female	353.1	Not available	Euthanized	Suspect mass in abdomen	Chronic lymphocytic enteritis	106.7
8	Female	305.6	2346	Euthanized	Suspected CLE, wasting and poor health	Lymphoma & Ulcerative enteritis	1540.0
9	Female	307.0	5170	Euthanized	Suspect E Coli infection	Enteropathogenic *E. Coli*	24.4
10	Female	302.0	3796	Euthanized	Doing poorly	Adenocarcinoma	111.4
11	Male	456.0	3654	Euthanized	Chronic diarrhea	Chronic lymphocytic enteritis	256.7

Table 32. Continued

Marmoset Number	Sex	Body Weight (g)	Age in days	Cause of death	Clinical history	Post mortem diagnosis based on gross and microscopic examination	Fecal NMH (µg/g)
12	Female	400.5	3838	Euthanized	Chronic diarrhea	Chronic lymphocytic enteritis	236.5
13	Male	272.9	6304	Euthanized	Weight loss	Renal failure	83.4
14	Male	357	4667	Euthanized	Renal failure	Renal failure	23.8

NMH – N-methyl histamine

had fecal NMH concentrations greater than the upper limit of the reference interval, and 6/7 of these had CLE. For diagnosis of CLE from a single fecal sample using a diagnostic cutoff of ≤ 118.2 ng/g the sensitivity of this assay is 86% (95% confidence interval: 42% to 100%), and the specificity of 86% (Range 42% to 100%; 95% confidence interval).

Of these 14 marmosets, extra tissue was only available for 6 for mast cell counting. Their details and the counts are shown in Table 33. Given the small number of animals, no statistical analysis was done.

7.4 Discussion

CLE is a common disease in marmoset colonies. A minimally invasive test to detect CLE would be beneficial in the common marmoset. Fecal NMH concentrations were increased in 7/8 common marmosets with CLE suggesting that this test may have diagnostic utility.

Based on the limited number of samples tested, the linearity and the accuracy of NMH GC/MS assay seems adequate for the assessment of marmoset fecal samples. The linearity and accuracy seem comparable to when this assay was used for measurement of NMH in dog fecal samples (dilutional parallelism O/E: 88.6% to 115.0%; mean spiking recovery O/E: 104.2%).[133]

The concentrations of NMH detected in fecal extracts ranged from undetectable to 216.1µg/g of feces. Undetectable NMH concentrations have also been reported in the dog.[133] The reference intervals established for the common marmoset fecal samples are comparable to canine reference intervals of ≤191 ng/g feces and ≤334 ng/g feces, for the 3-day mean and maximum fecal NMH concentrations, respectively.[13] To account for variations in fecal NMH concentrations, 3-day mean or 3-day maximum concentrations have been used in other studies,[13] and may also be

Table 33. Distribution of mast cells in the gastrointestinal tract of 6 marmosets from the NEPRC. Reprinted with permission.[118]

Marmoset Number	Necropsy diagnosis	Mast cells in four 20x fields from each slide				
		Stomach	Small Intestine			Large Intestine Cecum/ Colon
			Duodenum	Jejenum	Ileum	
1	Normal control	42	14	4,14	NA	7,3,6[a]
2	Normal control	NA	11	27,46	7,21[b]	3,21[b]
4	Chronic lymphocytic enteritis	169	12	51,21	93	38,0,23
5	Chronic lymphocytic enteritis	NA	23	110,78	109	47,69
10	Adenocarcinoma	NA	NA	26,38	NA	5,21
13	Renal failure	33	NA	43	NA	46, 57, 82

NA= Not available; numbers separated by commas suggest multiple slides were available.

Superscripts next to numbers indicated less than 4 fields were counted; a = 3 fields, b = 2 fields

more appropriate in the common marmoset given the intra-individual variation observed in this study.

The observed increases in fecal NMH concentrations for marmosets with CLE compared to controls and estimated diagnostic sensitivity and specificity suggest that this may have utility for the non-invasive diagnosis of this disease in marmosets. However, further studies with a larger number of animals are needed. Currently, the gold standard for diagnosis is post mortem examination and it is difficult to establish a definitive diagnosis ante-mortem. Therefore, a non-invasive test may allow earlier and more accurate diagnosis of this common disease. Fecal NMH was normal in one marmoset with CLE, this could represent a separate subtype of the disease, severity, issues with sample handling, or because of the intra-individual variation observed in the study. Increased concentration of fecal NMH was also observed in a case of lymphoma and ulcerative enteritis and which was unexpected. Increased mast cell activation currently known as 'mast cell activation syndrome' has been reported with chronic inflammatory or neoplastic disorders[3]. It is uncertain what leads to this increase and unfortunately no histological sections were available for evaluation of mast cells numbers in the gastrointestinal tract of these animals. Previously fecal NMH concentrations were also shown to be increased in some dogs with chronic enteropathies.[6,13] These studies also showed that fecal NMH was increased more in dogs with moderate intestinal inflammation, and only sometimes in dogs with mild inflammation.

Canine and human studies have used urine NMH to creatinine ratios as indicators of active disease,[6,13,163] or endoscopically diagnosed disease.[166] In our study, feces was used because the samples were already available, and were easier to collect. Some authors also believe that histamine concentrations in stool do not reflect a person's systemic burden but luminal active

histamine,[69] and hence feces may be a better marker of mast cell activation in CLE, and this is another reason why fecal samples were used rather than urine for our study.

To the best of our knowledge mast cells have not been described in the marmoset gastrointestinal tract. Previous studies evaluating mucosal mast cell numbers in dogs[76] and humans have reported mixed results. Staining techniques particularly employing toluidine blue may not be accurate as it only stains intact mast cell granules, whereas immunohistochemical stains targeting mast cell tryptase also react with residual enzymes from degranulated cells. Metachromatic stains are significantly cheaper than immunohistochemical staining and that is the reason we decided to pursue this in our study. A patchy distribution of mast cells throughout the gastrointestinal tract, non-representative biopsies, or ongoing degranulation of mast cells resulting in their depletion have also been reported as potential causes why mast cell counts may not be true representation of the disease process.[13] These could potentially explain the variation seen in the mast cell counts in the same location from the same marmoset in our study. In one healthy animal, mast cells reported in the colon were 3 and 21, and in a marmoset with CLE, they were 38, 0, and 23, raising concerns for a patchy distribution. However, no conclusions can be made, based on these findings, given the small number of animals.

Given that all the necropsy samples were from the same institution (NEPRC), and primate necropsies and histology are a common practice performed by boarded anatomic pathologists, no attempts were made to reevaluate the diagnosis of CLE in these samples. A report from a study in dogs[6] suggested that increased numbers of intestinal mast cells were seen in dogs responding to diet and/or antimicrobial therapy compared to immunosuppressive medications. This would have been valuable information if any such increases were detected in our study as it would help to guide treatment in the marmosets, unfortunately this could not be done due to low sample numbers.

The major limitation in this study was the small sample size (n=30) for establishing the reference interval, as well as the small number of animals (n=14) that underwent a necropsy and could subsequently be used to estimate diagnostic accuracy. No laboratory data were available to see if these animals also had low albumin concentrations or were anemic as described in previous studies. Given the small number of samples (n=6) available for specific staining of mast cell, no association between presence of mast cells and NMH concentrations could be demonstrated. Optimum fecal sample handling and storage conditions were also not assessed in this study. Since fecal samples were collected in the necropsied animals after death, it is unknown how much that could have an effect on the fecal NMH concentrations.

It is also possible that marmosets with increased fecal NMH concentrations and CLE demonstrated on histology were terminally ill and hence euthanized. Hence, the utility of fecal NMH in healthy colonies, or as an early marker of CLE remains uncertain.

Even with the given the limitations, the study however does suggest that common marmosets with chronic lymphocytic enteritis may have increased fecal NMH concentrations. Further studies in a larger number of common marmosets are warranted to better evaluate the role that mast cells play in chronic lymphocytic enteritis and to fully evaluate the utility of this test.

CHAPTER VIII

SUMMARY AND CONCLUSIONS

The common marmoset (*Callithrix jacchus*) is a New World primate that is being used extensively in biomedical research. Chronic diseases of the gastrointestinal tract, particularly chronic lymphocytic enteritis (CLE), is frequently observed in captive common marmoset research colonies. CLE is characterized by weight loss or a failure to thrive, hypoproteinemia, and anemia in affected marmosets and is often associated with death. To date, no single etiology has been identified and a multifactorial pathogenesis has been hypothesized. Ante-mortem diagnosis is challenging, as it requires ruling out other causes of gastrointestinal disease and other chronic diseases that maybe associated with similar clinical signs. A definitive diagnosis is made by confirmation of intestinal inflammation upon histopathology, which is often achieved during necropsy. Lesions are also observed in other organs such as the pancreas, liver, kidneys, and others. CLE is highly prevalent in research colonies, with a prevalence reaching as high as 60% in some colonies. Though recent reports suggest that the disease presentation is changing, CLE still poses a challenge for biomedical research using the common marmoset. Early identification of marmosets predisposed to CLE or those in the early stages of disease would help researchers using marmosets by facilitating their exclusion, particularly from long-term projects such as aging studies.[11]

To date, no single serum or fecal biomarker evaluated has proven useful in the diagnosis of CLE in marmosets. Also, as no etiology has been identified, no effective treatment exists. The hypoalbuminemia associated with CLE in marmosets is presumed to be from enteric protein loss due to intestinal inflammation. Fecal concentrations of alpha$_1$-proteinase inhibitor (α_1-PI) have

been used to diagnose enteric protein loss in humans, dogs, and cats with gastrointestinal disease. However, immunoassays for the measurement of α_1-PI in feces are species specific and no cross-immunoreactivity was observed evaluating marmoset serum in human, cat, or dog assays. Alpha$_1$-PI has not been previously studied in marmosets with CLE. Thus, this study was to determine if α_1-PI is clinically useful as a biomarker of enteric protein loss and as a marker of intestinal inflammation in the common marmoset. Other serum and fecal markers of intestinal inflammation such as serum cobalamin and folate concentrations and fecal N-methyl histamine were also evaluated.

8.1 Summary

As a prelude to immunoassay development marmoset α_1-PI was purified. Marmoset serum was used to purify α_1-PI using immunoaffinity chromatography and ceramic hydroxyapatite chromatography. Partial characterization was achieved by determination of molecular weight (54 kDa), relative molecular mass (51,677), and isoelectric point (4.8-5.4). Protein identity was confirmed with peptide mass fingerprinting and N-terminal amino acid sequencing (EDPQGDAAQKMDTSHH). Inhibitory enzyme activity of marmoset α_1-PI for trypsin, chymotrypsin, and elastase was determined. The overall yield of marmoset α_1-PI from serum was 12% using this method. Purified marmoset α_1-PI has structural and biochemical characteristics similar to those reported for α_1-PI in other species. Polyclonal antibodies were raised in New Zealand white rabbits by inoculation of purified marmoset α_1-PI using a commercial antibody production service. The specificity of the antibodies raised was confirmed by using a Western blot. Cross-reactivity of polyclonal antibody sera raised were tested against serum from several other

species using radial double immunodiffusion. Cross-immunoreactivity was only observed with serum from the Geoffrey's marmoset.

Pure marmoset α_1-PI was labelled with radioactive iodine (^{125}I) to generate a tracer. A competitive double antibody radioimmunoassay (RIA) for the measurement of serum and fecal concentrations of marmoset α_1-PI was successfully set up. The established RIA was analytically validated by determination of sensitivity, working range, dilutional parallelism, spiking recovery, and intra- and interassay variability and was found to be sensitive, linear, accurate, precise, and reproducible for the quantification of mα_1-PI concentrations in serum and fecal extracts. A reference interval for mα_1-PI concentrations in serum and feces was established in healthy marmosets.

The use of a radiolabel [^{125}I] in radioimmunoassay is a major drawback of radioimmunoassays, and with the short shelf life of the tracer,[53] enzyme immunoassays might be preferable. Hence, a sandwich enzyme-linked immunosorbent assay (ELISA) for the measurement of α_1-PI concentrations in serum and fecal extracts from the common marmoset was developed. Analytical validation of the ELISA was achieved by determining the lower limit of detection, linearity, accuracy, precision, and reproducibility. The ELISA was linear, accurate, precise, and reproducible for quantification of marmoset α_1-PI concentrations in serum and fecal extracts, however, with limited linearity and accuracy for spiking recovery of fecal samples. Reference intervals for serum and fecal α_1-PI concentrations in the common marmoset were established.Given the limited linearity and accuracy for measuring α_1-PI concentrations in fecal extracts when using the ELISA, concentrations of α_1-PI measured in the same fecal extract on both assays were compared using a Bland–Altman plot. Given the proportional bias identified between the RIA and the ELISA, the RIA was used for clinical validation. Fecal α_1-PI concentrations were

determined from fecal samples collected at necropsy from healthy marmosets, those with CLE, and those with other diseases. This study showed that fecal α_1-PI concentrations are not elevated in common marmosets with CLE. Though this was a study with a small sample size, and other limitations were present, it does suggest that other mechanisms might be responsible for the hypoalbuminemia seen in marmosets with CLE. Decreased serum albumin concentrations can also be caused by dietary protein deficiency, malassimilation, protein losing nephropathy, and chronic hepatopathy. Lesions in the kidney, pancreas, and liver are common concurrent necropsy findings in marmosets with CLE.[88] Albumin has been reported to be a negative acute phase protein in humans,[66] rhesus macaques,[82] and marmoset.[135] It is possible that the hypoalbuminemia seen in CLE is a result of chronic systemic inflammation, rather than the presumed enteric protein loss. The use of corticosteroids in marmosets with clinical signs of CLE did improve serum albumin concentrations.[115] Impaired digestive efficiency in marmosets with CLE[68] also may contribute to the low albumin levels. Larger studies evaluating fecal samples from marmosets with CLE at different time points are needed to confirm these results.

Serum cobalamin and folate concentrations can serve as surrogate markers of gastrointestinal disease in dogs and cats where they can have both therapeutic and prognostic implications. CLE in the common marmoset, still presents a diagnostic challenge and given the frequency that it was observed in captive common marmosets, a diagnostic test is desperately needed. Cobalamin and folate concentrations can be measured in serum using an automated chemiluminescent immunoassay. The current study validated a commercially available assay for the measurement of serum cobalamin and folate in the common marmoset, established a reference interval in healthy marmosets, and then measured serum concentrations of cobalamin and folate

in common marmosets with CLE. The commercial assay was linear, accurate, precise, and reproducible for the measurement of serum cobalamin and folate in the common marmoset. The study also showed that decreased serum folate concentrations were moderately sensitive (>70%) for CLE and decreased serum cobalamin concentrations were moderately specific (>70%) for CLE. Clinical implications of decreased serum cobalamin and folate concentrations in the common marmoset are yet to be determined.

Measurements of serum cobalamin and folate concentrations may aid in the diagnosis of CLE or gastrointestinal disease in general in the marmoset. However marmosets are rather small and there are limitations on serum samples that can be collected for evaluation. A naturally voided sample of feces or urine used for testing might facilitate easier screening of marmosets for CLE. Fecal N-methylhistamine (NMH) concentrations, a breakdown product of histamine released from degranulating mast cells, is increased in dogs with chronic enteropathy. The role of mast cells has not previously been evaluated in marmosets with CLE. Fecal NMH concentrations were measured using a gas chromatography–mass spectrometry assay that had previously been developed for use with canine fecal extracts. A partial validation was performed. Fecal NMH concentrations were measured in 14 marmosets, 7 of 8 marmosets with CLE and one marmoset with lymphoma and ulcerative enteritis had increased fecal NMH concentrations. Increased fecal NMH concentrations may serve as a potential marker for CLE. However, further studies exploring the role of mast cells in CLE in the common marmoset are needed.

8.2 Future directions

One of the major hypothesis for our study was that hypoalbuminemia seen in marmosets with CLE is a result of enteric protein loss. Our study failed to demonstrate increased enteric

protein loss, based on normal fecal α_1-PI concentrations in marmosets with CLE. Hence, another test to see if PLE is a component of CLE in marmosets is indicated. The 'gold standard test' in other species is the demonstration of fecal clearance of intravenously administered ^{51}Cr or ^{99m}Technetium - labeled human serum albumin. Testing this in a small population of terminal marmosets with CLE in the future would further help confirm or refute the role of PLE, if any, in CLE.

This study demonstrated abnormal serum concentrations of cobalamin and folate in marmosets with CLE, both of which were however only moderately sensitive and specific for the detection of CLE or gastrointestinal disease in the common marmoset. It should be noted that the diet of the marmosets in this study contained cobalamin. However, the amount of cobalamin contained in the diet was unknown. It would be interesting to determine the exact amount of cobalamin in the diet fed to marmosets to determine whether this potentially could mask cobalamin deficiency due to CLE in some of the animals. Also, serum cobalamin concentrations can be a poor marker of cellular cobalamin status, and a follow up test quantifying homocysteine, methylmalonic acid, and holo-transcobalamin may prove useful to further evaluate cobalamin status in these marmosets. Future studies should explore these cellular markers and this may potentially lead to tests with better sensitivity and specificity for the detection of CLE. In some human patients methylmalonic acid concentrations increase even before serum cobalamin concentration decreases, making it an earlier marker, which would be beneficial in research or breeding colonies of marmosets. Urinary concentration of methylmalonic acid can also serve as a marker of cobalamin deficiency in humans, and could serve as a non-invasive method of colony screening or testing. Hence, there is a significant potential for exploring these markers in future studies.

Our study showed for the first time that mast cells are present in the gastrointestinal tract of marmosets and increased fecal concentrations of N-methylhistamine, a marker of mast cell degranulation was increased in a portion of marmosets with CLE. Future studies should evaluate a larger number of healthy marmosets and marmosets with CLE, comparing serum, urinary, and fecal concentrations of N-methylhistamine to determine if the increase in the fecal concentrations is a result of systemic mast cell activation or a local process within the gastrointestinal tract.

Mast cells have also been shown to release metalloproteinase-9, another marker that has been reported previously to be elevated in marmosets with clinical signs comparable with CLE. This finding, along with our findings of elevated fecal NMH, does suggest a bigger unexplored role for mast cells in CLE. Histamine release is not unique to mast cells, as basophils also carry histamine granules. Eosinophil activation can also lead to increased histamine release. As mast cells, basophils, and eosinophils express many of the same receptors and cytokines, evaluation of specific mast cell markers such as tryptase in feces may be more useful in future studies. Further confirmation of mast activity in a larger number of marmosets with CLE by immunohistology staining for tryptase or by using transmission electron microscopy looking for evidence of degranulation is also warranted. This should be complemented by comparing levels of proinflammatory cytokines (e.g., IL-4 and IL-6), other substances synthesized and stored in mast cells granules (e.g., β-hexosaminidase activity and serotonin), and transcriptional markers (e.g., mRNA levels of MMP9) in gastrointestinal tissue or contents between healthy marmosets and marmosets with CLE.

The confirmation of mast cell activation as a potential driver of marmoset CLE would help target the management of affected animals, particularly by advocating the use of mast cells

stabilizers, such as oral cromolyn sodium, ketotifen, or natural bioflavonoids, such as quercetin, luteolin, or similar pharmaceuticals.

8.3 Conclusions

In conclusion, common marmosets with CLE do not have increased levels of fecal α_1-PI concentrations, suggesting that other factors may be contributing to hypoalbuminemia observed in these marmosets. In contrast, similar to other species, marmosets with gastrointestinal disease show decreased serum concentrations of folate and cobalamin. Also, fecal NMH concentrations are increased in marmosets with CLE, suggestive of mast cell involvement in the inflammatory process in the gut. Further studies are needed to determine the exact role of mast cells in the pathogenesis of CLE in marmosets to help direct therapeutic interventions, to improve treatment outcome, and decrease the prevalence of CLE in common marmosets used for biomedical research.

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